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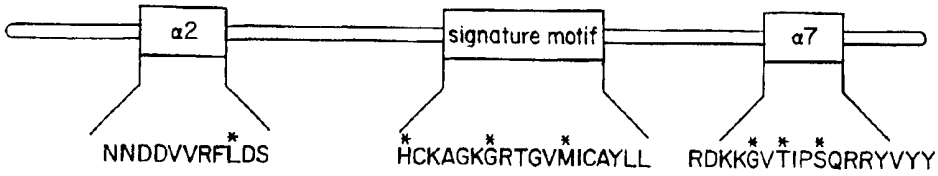

(57) **Abstract**

PTEN proteins and altered PTEN proteins, and the nucleic acid molecules encoding them are described. Also described are methods of diagnosis and treatment, e.g., of prostate cancer, utilizing compositions comprising PTEN or altered PTEN or nucleic acid molecules encoding PTEN or altered PTEN.

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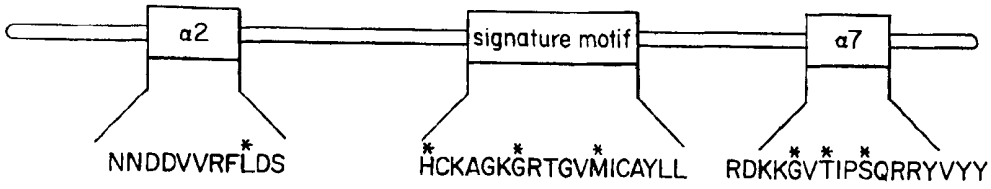

(57) Abstract

PTEN proteins and altered PTEN proteins, and the nucleic acid molecules encoding them are described. Also described are methods of diagnosis and treatment, e.g., of prostate cancer, utilizing compositions comprising PTEN or altered PTEN or nucleic acid molecules encoding PTEN or altered PTEN.

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DUAL SPECIFICITY PHOSPHATASE AND METHODS OF USE

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RELATED APPLICATIONS

This application claims the benefit of Provisional Application entitled "PTEN is a Functional Antagonist of PI 3-Kinase" by Nicholas K. Tonks and Michael P. Myers filed on June 29, 1998 and Provisional Application 60/051,908 filed July 8, 1997, the teachings of which are incorporated herein in their entirety.

BACKGROUND OF THE INVENTION

A variety of techniques have been used to identify genes involved in the etiology of cancer. From these studies, a surprisingly large number of protein tyrosine kinases (PTKs) have been implicated in carcinogenesis (Rosen N. *Molecular Basis of Cancer*, W.B. Saunders, pp. 105-140, (1995)). The PTKs are activated by amplification, deletion or mutation of important negative regulatory domains, or by genetic rearrangements which result in the production of activated fusion proteins (Rosen N. *Molecular Basis of Cancer*, W.B. Saunders, pp. 105-140, (1995); Gauwerky & Croce, *Molecular Basis of Cancer*, W.B. Saunders, pp. 18-37 (1995)). Further support for the importance of tyrosine phosphorylation in oncogenesis comes from the finding that expression of v-crk, a small adaptor protein which does not contain intrinsic PTK

activity, results in an increase in the levels of cellular phosphotyrosine and cellular transformation (Barker, K & Hanafusa, H. *Mol. Cell. Biol.* 10:3813-3817, (1990)). Although PTPs have been linked to the inhibition of cell proliferation, there have been no clear cut examples of these enzymes functioning as tumor suppressors.

All PTPs contain the catalytic signature motif HCXXGXXRS/T (Charbonneau, H. and Tonks, N.K., *Ann. Rev. Cell. Biol.* 8:463-493 (1992)). The cysteine residue in this motif is absolutely required for catalysis, as it acts as a nucleophile to attack the phosphorous atom in the phosphate moiety of its substrate, forming a thiol-phosphate intermediate (Barford, D., Flint, A.J. & Tonks, N.K. *Science.* 263:1397-1404 (1994)). Mutation of this cysteine to serine or alanine results in the complete loss of phosphatase activity (Flint, A.J. *et al. Proc. Natl. Acad. Sci. USA.* 94:1680-1685 (1997)). The dual specificity phosphatases, which catalyze the hydrolysis of phospho-seryl, -threonyl and -tyrosyl residues, also contain the canonical PTP catalytic motif (Sun, H., *et al. Cell.* 75:487-493 (1993)). However, presence of the PTP motif does not guarantee that the protein is a protein phosphatase, as demonstrated by the discovery of CEL-1, an RNA capping enzyme (Takagi, T, *et al. Cell.* 89:867-873 (1997)).

Recent data indicate that the PTPs exhibit a great deal of substrate specificity *in vivo* and are not simply unregulated antagonists of the signals mediated by the PTKs (Flint, A.J. *et al. Proc. Natl. Acad. Sci. USA.* 94:1680-1685 (1997), Garton, A.J., *et al. Mol. Cell. Biol.* 16:6408-6418 (1996)). In fact, PTPs can exert both positive and negative effects on signaling pathways, indicating that they do not simply function as "off switches" (Hertog, J, *et al. EMBO J.* 13:3020-3032 (1994)). In spite of the large number of PTKs that have been shown to play a role in oncogenesis (Rosen N. *Molecular Basis of Cancer*, W.B. Saunders, pp. 105-140, 1995)), there are no examples of PTPs which function as classical tumor suppressors.

Since their discovery, protein tyrosine phosphatases (PTPs) have been thought to play a role in tumor suppression due to their ability to antagonize the growth promoting protein tyrosine kinases. Recently, a candidate tumor suppressor gene, from human

chromosome 10q23, called PTEN or MMAC1, has been identified that shares homology with the protein tyrosine phosphatase family (Steck, P.A. *et al. Nature Genetics.* 15:356-362 (1997), Li, J. *et al. Science* 275:1943-1946 (1997)). The chromosomal locus 10q22-23 is deleted in a large number of tumors, especially glioblastoma (70%) and prostate tumors (30%) ((Steck, P.A., *et al. Nature Genet.* 15:356-362 (1997), Li, J., *et al. Science.* 275:1943-1946 (1997)). PTEN is deleted in a large number of tumors, and germline mutations in PTEN give rise to a number of related neoplastic disorders, including Cowden's disease, an inherited disorder typified by the formation of multiple, benign tumors (hamartomas) in multiple tissues and an increased susceptibility to some forms of malignant cancer (Liaw, D. *et al. Nature Genetics* 16:64-67 (1997), Marsh, D.J. *et al. Nature Genetics 16*:333-334 (1997), Nelen, M.R. *et al. Hum. Mol. Gen. 6*:1383-1387 (1997) and Marsh, D.J. *et al. Hum. Mol. Genet. 7*:507-515 (1998)). Mutations in PTEN were also found in patients suffering from Lhermitt-Duclos disease, a variant of Cowden's Syndrome, which has additional pathologies, including ataxia, macrocephaly and dysplastic cerebellar gangliocytomatosis (Mallory, S.B. *Derm Clinics* 13:27-31 (1995)).

SUMMARY OF THE INVENTION

PTEN, a candidate tumor suppressor gene identified on chromosome 10, shares homology with the PTP family, as well as with the cytoskeletal protein tensin (Steck, P.A., *et al. Nature Genet.* 15:356-362 (1997), Li, J., *et al. Science.* 275:1943-1946 (1997)). As described herein, purified PTEN showed a preference for acidic substrates. Initial enzymatic characterization of PTEN (Myers *et al. Proc. Natl. Acad. Sci USA 94*:9052-9057 (1997)) revealed specificity for highly acidic substrates *in vitro*. This observation led to the consideration of signaling molecules with acidic characteristics as potential substrates and focused our attention on the phosphoinositides. As described herein, PTEN dephosphorylates phosphatidylinositol *in vitro*. It is also demonstrated that PTEN displays selectivity for the 3 position of the inositol ring, unlike the known

lipid phosphatases which are specific for the 5 position. Furthermore, as also shown herein, PTEN is able to dephosphorylate serine, threonine and tyrosine residues when present in acidic substrates. Phosphatase activity of PTEN is necessary for its ability to function as a tumor suppressor or an apoptosis inducer. A variety of point mutations, including point mutations identified in tumor samples and Cowden's disease kindreds, ablated PTEN activity.

The function of PTEN as a phosphatidylinositol phosphatase has been examined in a physiological context in work described herein. Firstly, expression of wild type PTEN in 293 cells suppresses the levels of phosphatidylinositol (PtdIns $(3,4,5)P_3$), whereas expression of a "substrate-trapping" mutant form of the enzyme leads to accumulation of the phospholipid. These data illustrate that PTEN recognizes phosphatidylinositol triphosphate as substrate in a cellular context. Secondly, in tumor cell lines that are defective in expression of PTEN, ectopic expression of wild type PTEN results in suppression of the phosphorylation of PKB/Akt, a downstream target of the lipid products of phosphatidylinositol-3-OH kinase (PI 3 kinase). Furthermore, phosphorylation of the pro-apoptotic protein BAD, a substrate of PKB/Akt, was also suppressed in cells ectopically expressing wild type PTEN. Although it was possible to achieve expression of inactive mutant forms of PTEN in the prostate cancer cell line LnCaP, viable cells expressing the wild type enzyme could not be recovered. However, this effect of PTEN could be rescued by expression of a constitutively-active, membrane-targeted form of PKB/Akt. These data reveal that PTEN functions as an upstream, negative regulator of PKB/Akt and has the potential to regulate signals associated with control of cell survival.

These results provide very important insights into the physiological function of PTEN as a phosphatidylinositol phosphatase. This work reveals a novel mechanism by which a tumor suppressor phosphatase may regulate cell growth and also reveals an important cancer diagnostic or cancer therapy target. These results also highlight the

fact that members of the dual specificity class of protein tyrosine phosphatases may recognize non-proteinaceous targets *in vivo*.

Thus, the present invention relates to PTEN, and isolated nucleic acid sequences encoding PTEN, having phosphatase activity against phosphorylated non-proteinaceous substrates, tyrosine residues, phosphorylated serine residues, phosphorylated threonine residues, and combinations thereof. In one embodiment, PTEN dephosphorylates PtdIns $(3,4,5)P_3$. In another particular embodiment, PTEN dephosphorylates phosphorylated tyrosine, phosphorylated serine or phosphorylated threonine residues present in substrates containing acidic residues. The invention also relates to altered forms of PTEN, and isolated nucleic acid sequences encoding the altered forms of PTEN, which have altered phosphatase activity against PtdIns $(3,4,5)P_3$ phosphorylated tyrosine, phosphorylated serine or phosphorylated threonine residues.

The present invention further relates to use of the described nucleic acid sequences, their encoded gene products, altered forms of the nucleic acid sequences and encoded gene products, and antibodies that recognize or bind to PTEN or specific altered PTEN phosphatases. These compositions can be used to assess the presence or activity of PTEN or altered PTEN as described herein. These compositions can also be used in the diagnosis and treatment of disease, particularly in the diagnosis and treatment of conditions characterized by an alteration in PTEN which causes an alteration of phosphatase activity. In a particular embodiment, the alteration in PTEN results in an inhibition of phosphatase activity or an alteration (e.g., increase) in PTEN-induced apoptosis. In another embodiment, the alteration in PTEN results in an enhancement of phosphatase activity.

The present invention relates to an isolated nucleic acid sequence encoding PTEN phosphatase, wherein the encoded PTEN phosphatase dephosphorylates PtdIns$(3,4,5)P_3$. The present invention also relates to an isolated nucleic acid sequence encoding PTEN phosphatase wherein the encoded PTEN phosphatase dephosphorylates phosphorylated tyrosine residues, phosphorylated serine residues, phosphorylated

threonine residues and combinations thereof. The present invention also pertains to an isolated nucleic acid sequence encoding PTEN phosphatase, wherein the encoded PTEN phosphatase possesses substrate sensitivity such that the phosphatase is active against acidic substrates or the activity is enhanced by presence of acidic amino acid residues in a substrate of the PTEN phosphatase. The present invention also pertains to an isolated nucleic acid sequence encoding an altered phosphatase, wherein the phosphatase has an alteration in amino acid sequence such that phosphatase activity is inhibited (e.g., reduced or abolished). In one embodiment, the alteration in amino acid sequence is in the catalytic domain of the phosphatase. The isolated nucleic acid sequence encoding the phosphatase can have an alteration wherein the alteration is a point mutation. For example the alteration can be a point mutation resulting in alteration of histidine 123 to tyrosine, alteration of glycine 129 to arginine or alteration of glycine 129 to glutamic acid. The present invention is also drawn to alterations in amino acid sequence wherein the alteration occurs outside of the catalytic domain. The alteration, such as a point mutation, can occur within alpha helix 2 or alpha helix 7 of the phosphatase. The point mutation can result in alteration of serine 170 to arginine, alteration of glycine 165 to arginine, or alteration of threonine 167 to proline.

The present invention further relates to an isolated PTEN phosphatase, wherein the phosphatase dephosphorylates acidic or lipid substrates such as PtdIns(3,4,5)P_3. The present invention also relates to an isolated PTEN phosphatase wherein the phosphatase dephosphorylates phosphorylated tyrosine residues, phosphorylated serine residues, phosphorylated threonine residues and combinations thereof. The present invention relates to an isolated PTEN phosphatase wherein the phosphatase possesses substrate sensitivity such that phosphatase is active against acidic substrates or the activity is enhanced by presence of acidic amino acid residues in a substrate. The present invention also pertains to an isolated altered phosphatase having an alteration in amino acid sequence such that phosphatase activity is inhibited.

The present invention also relates to an antibody that specifically binds to PTEN, or a portion or altered form of PTEN, as well as to antigen binding fragments of such antibodies.

The present invention further relates to methods of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual, wherein nucleic acids in a sample of cells from the individual are combined under conditions appropriate for hybridization with a nucleic acid probe which hybridizes with nucleic acids encoding an altered PTEN or portion thereof, such as an alteration which causes an alteration of phosphatase activity of PTEN. Hybridization of nucleic acids in the sample of cells with the nucleic acid probe is detected, and hybridization of nucleic acids in the sample of cells with the nucleic acid probes is indicative of a condition characterized by an alteration in PTEN. In one embodiment, the nucleic acid probe detects an alteration in the catalytic domain, alpha helix 2 or alpha helix 7 of PTEN. In another embodiment, the alteration of phosphatase activity is inhibition of phosphatase activity. In one embodiment of the present invention, the condition is tumorigenesis.

The present invention further relates to a method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual. In one embodiment, proteins in a sample of cells from the individual are contacted with an antibody which binds altered PTEN. In one embodiment, the altered PTEN contains at least one amino acid which is different from the wild-type or reference amino acid and has altered phosphatase activity. Antibody binding to altered PTEN is detected, and binding of the antibody to altered PTEN is indicative of a condition characterized by an alteration in PTEN. In one embodiment, the specific antibody detects an altered PTEN wherein portions of the carboxy terminus of PTEN have been deleted, or wherein PTEN is altered in the catalytic domain, or wherein PTEN is altered in alpha helix 2 or alpha

helix 7. In one embodiment, the alteration of phosphatase activity results in inhibition of phosphatase activity. In one embodiment, the condition is tumorigenesis.

The present invention also pertains to a method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual. In one embodiment, proteins from the individual are combined with a phosphorylated PTEN substrate, under conditions appropriate for dephosphorylation of the phosphorylated substrate by PTEN. Presence or absence of dephosphorylation of the phosphorylated PTEN substrate is detected, wherein absence (including reduction relative to an appropriate control) of dephosphorylation is indicative of a condition characterized by an alteration in PTEN phosphatase activity.

In one embodiment, PTEN or altered PTEN is isolated from the proteins in a sample of cells from the individual and the phosphatase activity of the isolated PTEN or altered PTEN is measured. For example, proteins in a sample of cells from the individual can be contacted with a composition comprising specific antibody which binds PTEN or altered PTEN, under conditions appropriate for binding of the antibody to PTEN. The antibody-bound PTEN or altered PTEN can be isolated using methods described. In one embodiment of the present invention, the phosphorylated substrate is PtdIns(3,4,5)P_3. In another embodiment, the phosphorylated substrate is composed of acidic amino acids.

The present invention also relates to a method of treating a condition in an individual in which enhancement of PTEN phosphatase activity is desirable. In one embodiment, PTEN or a nucleic acid molecule encoding PTEN is administered in an appropriate physiologically acceptable vehicle to an individual. In one embodiment, the condition can be hyperproliferative diseases including brain, prostate or breast cancers, as well as Cowden's disease.

The present invention also relates to a method of treating a condition in an individual in which reduction of PTEN phosphatase activity is desirable. In one

embodiment, an altered form of PTEN or a nucleic acid molecule encoding an altered form of PTEN is administered in an appropriate physiologically acceptable vehicle to an individual. For example, the condition can be a degenerative disease including, but not limited to, Parkinson's and other neurodegenerative diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1C are the nucleotide sequence of the PTEN gene (SEQ ID NO: 1) and the predicted amino acid sequence (SEQ ID NO: 2). The predicted start codon for the amino acid sequence is at nucleotide position 490, generating a protein 403 amino acids in length.

Figures 2A-2C are graphic representations of results which show the proteinaceous substrate specificity of purified PTEN. PTEN was tested for protein phosphatase activity using the indicated tyrosine (Figure 2A) or serine/threonine (Figure 2B) substrates. Activity is expressed as pmol phosphate released. A catalytically inactive mutant of PTEN (PTENC124S) was included as a control to rule out the possibility of contaminating bacterial phosphatases. Figure 2C shows a comparison of PTEN and cdc14 activities. PTEN and cdc14 were assayed as above with maleylated lysozyme (RCML) or $polyGlu_4Tyr_1$, and the activity is expressed as pmol phosphate released/min/mg.

Figure 3 depicts the location of PTEN mutations. The diagram of PTEN shows the locations of the point mutations, which are indicated by an "*", that were tested as described herein. In addition, the predicted structural motif in which these mutations lie is indicated.

Figure 4 is a graphic representation of phosphatase activity assays (against proteinaceous substrates) which illustrates disruption of PTEN activity by point mutations found in tumor samples. The indicated point mutations were introduced into recombinant PTEN and their effects on phosphatase activity were determined. Assays were performed with $polyGlu_4Tyr_1$ for 15 minutes. Activity is expressed as pmol of

phosphate liberated/min/mg of PTEN. Assays were performed in triplicate and are expressed as the mean +/- the standard deviation. The catalytically inactive mutant of PTEN (PTENC124S) was included as control to rule out the possibility of contaminating bacterial phosphatases.

Figure 5A is a graph of the level of $^{32}P_i$ released by the indicated phosphatases.

Figure 5B is a graph of the rate of $^{32}P_i$ released from the indicated substrates by PTEN.

Figure 6 is a graph of the level of $^{32}P_i$ released by the indicated phosphatases transfected into HEK293 cells.

Figure 7 is a graph of the percentage of GFP positive LnCaP cells isolated after the cells were transfected with the indicated PTEN.

DETAILED DESCRIPTION OF THE INVENTION

PTEN, a tumor suppressor gene identified on chromosome 10, shares homology with the PTP family, as well as with the cytoskeletal protein tensin ((Steck, P.A., *et al. Nature Genet.* 15:356-362 (1997), Li, J., *et al. Science.* 275:1943-1946 (1997)). PTEN was isolated from a locus on chromosome 10, 10q22-23, which is deleted in a large number of tumors. Germline mutations in PTEN give rise to Cowden's disease and variants thereof, which are typified by the formation of multiple, benign tumors and an increased susceptibility to malignant cancers.

PTEN possesses the signature motif that defines the PTP family of enzymes (Steck, P.A. *et al. Nature Genetics* 15:356-362 (1997), Li, J. *et al. Science* 275:1943-1946 (1997)). Initial studies revealed that PTEN can function as a dual specificity phosphatase *in vitro* displaying selectivity for extremely acidic substrates (Myers, M.P. *et al. Proc. Natl. Acad. Sci. USA 94*:9052-9057 (1997)). Work described herein demonstrating the substrate selectivity of PTEN and the critical importance of PtdIns(3,4,5)P_3 as a second messenger in signaling events controlling cell proliferation suggested that PTEN may recognize this acidic phospholipid as a physiological.

Finally, as demonstrated herein, phosphatase activity of PTEN is necessary for its ability to function as a tumor suppressor, because a variety of point mutations, which are known to occur in tumors and certain Cowden's disease kindreds, ablated PTEN activity.

A key step in understanding the function of PTEN is to identify its physiological substrates. Work described herein indicates that the lipid second messenger molecule phosphatidyl inositol phosphate, which has been implicated in signaling events associated with cell growth, is a physiological substrate of PTEN. Expression of PTEN in HEK293 cells reduced the levels of the phospholipid products of PI 3-kinase. Furthermore, ectopic expression of the phosphatase in PTEN-deficient tumor cell lines resulted in the inhibition of PKB/Akt and the induction of apoptosis.

Glioblastoma is one of the most common and malignant forms of cancer, and is often characterized by the constitutive activation of EGF-dependent signaling pathways due to the amplification of members of the EGF receptor family of protein tyrosine kinases (PTKs). The products of tumor suppressor genes can attenuate these signaling pathways and their loss through deletion or mutation contributes to tumor progression. The 10q23 region of human chromosome 10 is frequently deleted or mutated in a wide variety of tumor types, most frequently in glioblastoma, endometrial cancer and prostate cancer, indicating the presence of a tumor suppressor gene at this locus. Recently, mutated PTEN has been identified in a number of tumor cell lines (Steck, P.A. *et al. Nature Genetics* 15:356-362 (1997), Li, J. *et al. Science* 275:1943-1946 (1997)). Importantly, germline transmission of mutations in PTEN gives rise to a related set of disorders, including Cowden disease, that are characterized by numerous small benign tumors and an increased incidence of other malignant growths (Liaw, D. *et al. Nature Genetics* 16:64-67 (1997), Marsh, D.J. *et al. Nature Genetics 16*:333-334 (1997), Eng, C. *et al. J. Med. Genet. 31*:458-461 (1994)). PTEN appears to be preferentially lost in advanced cancers, suggesting that its deletion is not the transforming event but that PTEN is inhibiting other cellular functions necessary for tumor progression (Whang,

Y.E. *et al. Proc. Natl. Acad. Sci. USA* 95:5246-5250 (1998), Rasheed, B.K. *et al. Cancer Res.* 57:4187-4190 (1997)).

The most common chromosomal deletion in glioblastoma occurs around 10q22-23, suggesting the presence of a tumor suppressor at this locus (Fults, D., *et al. Cancer Res.* 50:5784-5789 (1990)). Mapping of the deletions at 10q22-23, as well as Representational Difference Analysis (Lisitsyn N.A., *et al., Proc. Natl. Acad. Sci. USA.* 92:151-155 (1995)), led to the identification of PTEN as a candidate tumor suppressor residing at this locus (Steck, P.A., *et al. Nature Genet.* 15:356-362 (1997), Li, J., *et al. Science.* 275:1943-1946 (1997)). PTEN, also known as MMAC1 (Mutated in Multiple Advanced Cancers), shares sequence homology with the cytoskeletal protein tensin and with the family of PTPs (Steck, P.A., *et al. Nature Genet.* 15:356-362 (1997), Li, J., *et al. Science.* 275:1943-1946 (1997)). Approximately 70% of all glioblastoma samples tested have either deleted or mutated PTEN alleles. In addition, PTEN has been shown to be disrupted in a large number of breast and prostate tumor samples (Steck, P.A., *et al. Nature Genet.* 15:356-362 (1997), Li, J., *et al. Science.* 275:1943-1946 (1997)). Importantly, germline mutations in PTEN give rise to Cowden's disease, a disorder characterized by the formation of multiple benign tumors (hamartomas), as well as an increased susceptibility to malignant cancers of the ovary, breast, and thyroid (Mallory, S.B. *Derm. Clinics* 13:27-31 (1995)), suggesting that PTEN functions similarly to classical tumor suppressor genes (Liaw, D. *et al., Nature Genet.* 16:64-67 (1997)). It is noted that homologous genes isolated from *C. elegans* and *S. pombe* appear to have an extended 5' coding region that would result in a protein longer than that predicted by the human gene. There is also a potential, albeit unusual, start site in the human gene upstream of the predicted start site that would result in a longer protein.

As described herein, enzymatic activity of recombinant PTEN and the effects of mutations which occur in certain tumors and in Cowden's syndrome kindreds was assessed. Low specific activity of the fusion proteins was detected when assayed with proteins that are commonly used as PTP substrates, such as maleylated lysozyme

(RCML) or myelin basic protein (MBP). Recently, weak phosphatase activity was measured when RCML was used as a substrate (Li, D.-M. & Sun, H. *Cancer Res.* 57:2124-2129 (1997)). However, as demonstrated herein, PTEN exhibits robust phosphatase activity when assayed with a random copolymer of glutamate and tyrosine, polyGlu$_4$Tyr$_1$, suggesting that it exhibits an unusually high degree of substrate specificity. As used herein, the term "substrate specificity" describes the minimum level of interaction between phosphatase and substrate, such that ^{32}P is released at a minimum of 50 pmol/min/mg phosphatase under conditions described in the Examples. Also, as used herein, the term "acidic substrate" is intended to include polypeptides comprised of amino acids having acidic functional groups in addition to serine, threonine or tyrosine residues, such that the acidic functional groups are localized near the phosphate group in the three dimensional structure of the substrate, and non-proteinaceous substrates, such as PtdIns(3,4,5)P_3.

PTEN was shown herein to exhibit activity against serine/threonine, as well as tyrosine phosphorylated proteins (the Table and Figures 2A and 2B). PTEN showed a preference for substrates containing acidic residues. Even the best serine/threonine substrates, the ETE (RRREEETEEE, SEQ ID NO: 6) and DSD (RRRDDDSDDD, SEQ ID NO: 5) peptides, were not as efficiently dephosphorylated as polyGlu$_4$Tyr$_1$ (the Table and Figure 2A). Casein, phosphorylated by casein kinase II, was dephosphorylated to a lesser extent by PTEN (Figure 2B), suggesting that PTEN may require multiple acidic residues positioned both – and C-terminally to the phosphorylated residue.

However, as shown in Figure 5, PTEN dephosphorylates PtdIns(3,4,5)P_3 as efficiently as polyGluTyr, whereas a mutant PTEN (PTENC124S) in which the cysteine from the signature catalytic motif is replaced with serine, is unable to dephosphorylate PtdIns(3,4,5)P_3.

Mutations that occur in PTEN during tumorigenesis typically fall into three general classes: (i) genomic deletions encompassing all or most of PTEN, (ii) frame shift mutations resulting in the production of truncated PTEN proteins and (iii) point

mutations resulting in at least one substitution of an amino acid for another (Steck, P.A., *et al. Nature Genet.* 15:356-362 (1997), Li, J., *et al. Science.* 275:1943-1946 (1997)). As described herein, a variety of mutations found in tumor samples have been introduced into PTEN. Comparisons of PTEN with other phosphatases, whose crystal structures have been solved, aids in predicting how these point mutations might disrupt PTEN activity. For example, the mutation of histidine 123 to tyrosine (H123Y) results in complete loss of phosphatase activity against proteinaceous substrates mutation of glycine 129 to arginine (isolated from glioblastoma), results in the complete loss of phosphatase activity against proteinaceous substrates as well as PtdIns(3, 4, 5)P_3 (Table 1, Figure 5A). Although, glycine 129 is not as highly conserved as others found in the catalytic motif, the substitution of the large, charged side chain of arginine, a mutation found in a glioblastoma cell line, for the much smaller glycine, is likely to have a deleterious effect on the overall structure of the phosphate binding loop and is likely to impede the binding of phosphate.

Table 1: Specific activity of PTEN measured with tyrosine, serine and threonine phosphorylated substrates.

Substrate	Specific Activity (mean pmol phosphate released/min/mg enzyme)
Phosphotyrosyl Substrates	
$polyGlu_4Tyr_1$	4840(+/-140)
RCML	88(+/-6.6)
EDNDYINASL peptide	51(+/-4.0)
MBP	21(+/-1.2)
Phospho-seryl and -threonyl Substrates	210(+/-4.2)
DSD peptide	161(+/-1.8)
ETE peptide	14.2(+/-1.2)
Casein (CKII)	27.5(+/-2.2)
Casein (PKA)	11.3(+/-0.6)
MBP	

Several other mutations that reside outside of the catalytic motif were also tested for their effect on PTEN activity. A point mutation discovered in a glioblastoma sample that changes leucine 57 to tryptophan also eliminated PTEN phosphatase activity against proteinaceous substrates. This amino acid is located in a conserved alpha helix α2, which is found in both PTP1B and VHR. Although not directly involved in catalysis, this helix helps form the overall secondary structure of the enzyme (Stuckey, J.A. *et al. Nature* 370:571-575 (1994), Yuvaniyama, J. *et al. Science* 272:1328-1331

(1996)). Similarly, mutation of residues in this helix of LAR, a receptor PTP, also resulted in a significant loss of phosphatase activity (Streuli, M., *et al. Embo J.* 9:2399-2407 (1990)). A second cluster of point mutations was discovered in the last conserved structural motif found in most PTPs and dual specificity phosphatases, which also is an α-helix (Stuckey, J.A. *et al. Nature* 370:571-575 (1994), Yuvaniyama, J. *et al. Science* 272:1328-1331 (1996)). In YopH, a PTP isolated from the causative agent of bubonic plague, a hydrophobic residue in this α-helix is important for coordinating the water molecule necessary for regenerating the active enzyme (Yuvaniyama, J. *et al. Science* 272:1328-1331 (1996)). All three mutations found in this region: glycine 165 to arginine (found in a glioblastoma), threonine 167 to proline (found in a breast cancer) and serine 170 to arginine (found in a patient with symptoms of Cowden's Disease), resulted in the loss of PTEN activity against proteinaceous substrates. The G165R and the S170R mutations result in substitution of relatively small, uncharged amino acids for a much larger positively charged residue, potentially disrupting important interactions between this α-helix and surrounding structures. Moreover, the substitution of threonine 167 for proline is also likely to disrupt these interactions by interrupting the proper folding of this α-helix. These data indicate that this conserved helix (α7 in YopH and VHR) is a required motif in the dual specificity phosphatases.

Since all the mutations tested, with the exception of the M134L mutation, occur in cell lines or tumors where the opposing allele of PTEN has been deleted, these mutations are predicted to result in the complete loss of enzymatically active PTEN in the affected cells. The greatly reduced activity seen with the mutations tested, even those found well outside of the conserved catalytic motif, suggests that the inhibition of the enzymatic activity of PTEN was required for the progression of these cells to a cancerous state.

Most dual specificity phosphatases dephosphorylate and inactivate the MAP kinases (Sun, H., *et al. Cell.* 75:487-493 (1993), Kwak, S.P. *et al. J. Biol. Chem.* 269:3569-3604 (1994), Mourey, R.J., *et al. J. Cell Biol.* 271:3795-3802 (1996)), and

moreover, the MAP kinases are found to be hyperphosphorylated in breast cancer (Takagi, T, *et al. Cell.* 89:867-873 (1997)).), a cancer that frequently contains PTEN mutations or deletions, suggesting that the MAP kinases may be regulated by PTEN. Therefore, PTEN was tested for its ability to dephosphorylate the MAP kinase ERK2. However, as described herein, PTEN was incapable of dephosphorylating ERK *in vitro*, strongly suggesting that the MAP kinases are not regulated by PTEN *in vivo*.

Recombinant PTEN, produced in *E. coli* (Myers, M.P. *et al. Proc. Natl. Acad. Sci. USA 94*:9052-9057 (1997)), was assayed for its ability to release $^{32}P_i$ from radiolabeled PtdIns(3,4,5)P_3 or polyGluTyr (Figure 5A). Recombinant PTEN catalyzed the dephosphorylation of both substrates efficiently (Figure 5A). A catalytically-inactive mutant of PTEN (PTENC124S), in which the essential, catalytic cysteine from the signature motif was replaced with serine, was unable to dephosphorylate PtdIns(3,4,5)P_3 indicating that the lipid phosphatase activity was not due to a bacterial contaminant. In addition, cdc14, a dual specificity phosphatase closely related to PTEN, was also unable to dephosphorylate PtdIns(3,4,5)P_3, demonstrating that recognition of this phospholipid substrate is not a general property of other, even closely related, dual specificity phosphatases. PTEN also dephosphorylated inositol(1,3,4,5) tetrakisphosphate, the polar headgroup of PtdIns(3,4,5)P_3 but at a slower rate. To determine whether there was positional specificity in the PtdIns(3,4,5)P_3-directed phosphatase activity, lipid substrate labeled exclusively in the 3-position with ^{32}P was incubated with PTEN or SHIP, a well characterized 5-phosphatase (Lioubin, M.N. *et al. Genes Dev. 10*:1084-1095 (1996)). The products of these reactions were analyzed by thin layer chromatography. Incubation with SHIP yielded a radiolabeled product with the expected mobility of PtdIns(3,4)P_2. However, no labeled lipid products were generated following treatment with PTEN, under conditions where >50% of the ^{32}P was lost from the substrate (Figure 5B). The site selectively of PTEN was confirmed by HPLC analysis of similar reactions using [^{3}H- and 3-^{32}P]-labeled Ins(1,3,4,5)P_4. In addition to PtdIns(3,4,5)P_3 and Ins(1,3,4,5)P_4, PTEN also hydrolysed the other potential

products of PI 3-kinase, PtdIns(3)P and PtdIns(3,4)P_2, with the following rank order: PtdIns(3,4,5)P_3 = PtdIns(3,4)P_2 > PtdIns3P > Ins(1,3,4,5)P_4.

Previously, it has been shown that a point mutation in the PTP signature motif (PTEN-G129E), identified in two independent Cowden's disease kindreds, did not adversely effect the activity of PTEN toward protein substrates (Myers, M.P. *et al. Proc. Natl. Acad. Sci. USA 94*:9052-9057 (1997)). Remarkably, the activity of PTEN-G129E toward PtdIns(3,4,5)P_3 was reduced by approximately 90% relative to wild type enzyme (Figure 5A), while the activity was reduced against the proteinaceous substrate, polyGlu$_1$Tyr$_4$ by 25%. Another mutation (PTEN-G129R), isolated from a glioblastoma, in which the same glycine residue is changed to arginine, exhibited reduced activity when assayed with either PtdIns(3,4,5)P_3 or polyGluTyr. These data, demonstrating that a mutation found in Cowden disease specifically ablates the lipid phosphatase activity of PTEN, illustrate that the lipid phosphatase activity, rather than the protein phosphatase activity, is required for PTEN to function as a tumor suppressor. The specificity of PTEN for products of PI 3-kinase suggested that PTEN functions as a negative regulator of PI 3-kinase mediated signaling. A mutation identified in a prostate tumor cell line, in which methionine 134 was changed to leucine (M134L), had no effect on PTEN activity against proteinaceous substrates.

The potential for PTEN to antagonize PI 3-kinase signaling was investigated in HEK293 cells (Figure 6). Transient expression of PTEN lowered the levels of PtdIns(3,4,5)P_3. Importantly, PTEN-C124S, in which the catalytic cysteine has been mutated to serine, resulted in an increase in the levels of PtdIns(3,4,5)P_3 (Figure 6). The accumulation of PtdIns(3,4,5)P_3 in response to expression of PTEN-C124S is likely due to the ability of this form of PTEN to behave as a "substrate trapping" mutant, which yields a stable complex with the lipid substrate and protects it from dephosphorylation by endogenous phosphatases (Sun, H. *et al. Cell 75*:487-493 (1993)). The accumulation of PtdIns(3,4,5)P_3 in the presence of a substrate trapping mutant confirms that PtdIns(3,4,5)P_3 is a physiological target of PTEN ((Sun, H. *et al. Cell 75*:487-493 (1993), (Garton, A.J. *et al. Mol. Cell. Biol. 16*:6408-6418 (1996)). Expression of a

constitutively-activated PI 3-kinase (p110-CAAX) resulted in elevated levels of PtdIns(3,4,5)P_3 that were reduced approximately 60% following co-expression with PTEN and were increased approximately 4.5 fold when co-expressed with PTEN-C124S (Figure 6). The ability of PTEN to decrease PtdIns(3,4,5)P_3 levels produced by p110-CAAX further indicates that this phosphatase exerts its efforts on the products of PI 3-kinase rather than by dephosphorylating the tyrosine residues responsible for recruiting the p85-p110 PI 3-kinase complex to the membrane.

Signaling downstream of PtdIns(3,4,5)P_3 was also assessed by determining the phosphorylation status of PKB/Akt, a kinase whose activation is dependent upon the generation of PtdIns(3,4,5)P_3 or PtdIns(3,4)P_2 (Bos, J.L. *Trends Biochem. Sci. 20*:441-442 (1995)). Many tumor cell lines have been characterized which have lost PTEN expression through deletion or mutation of the endogenous gene (Steck, P.A. *et al. Nature Genetics* 15:356-362 (1997), Li, J. *et al. Science* 275:1943-1946 (1997)). Clonal cell lines were generated in two of these, U87 MG and U373 MG, in which PTEN expression was restored by infection with recombinant retroviruses. The parental cell lines exhibited high levels of phosphorylated PKB/Akt, whereas in every wild type PTEN-expressing cell line the levels of activated PKB/Akt were significantly lowered. Expression of a catalytically-inactive mutant of PTEN, PTEN-R130M, had no effect on basal PKB/Akt activation. Similarly, no effect on PKB/Akt activation was observed in a U373 MG (PTEN-SRVE #B9) clone infected with a defective virus. Following reconstitution of PTEN expression in the tumor lines, activation of PKB/Akt by insulin of PDGF was unaffected, illustrating that there are regulatory mechanisms which allow for the generation of PtdIns(3,4,5)P_3 following growth factor stimulation. The activation of PKB/Akt was completely inhibited by wortmannin in the tumor cell lines indicating that the increased PKB/Akt was PI 3-kinase-dependent.

One of the downstream substrates of PKB/Akt is the death effector protein BAD (Marte, B.M. and Downward, J. *Trends Biochem. Sci. 22*:355-358 (1997)). Phosphorylation of BAD by PKB/Akt promotes its dissociation from the BCL_{x1} and its association with 14-3-3, thus blocking the ability of BAD to induce apoptosis.

Therefore, phosphorylation of BAD promotes survival in cells expressing BCL_{x1} (Datta, S.R. *et al. Cell 91*:231-241 (1997)). Although, expression of PTEN in both U87 MG and U373 MG resulted in the reduction of BAD phosphorylation, we did not detect evidence of apoptosis in the PTEN-expressing clones. However, expression of wild type PTEN in LnCaP cells, a PTEN-deficient prostate cancer cell line, resulted in a decrease in the number of PTEN positive/GFP positive cells recovered relative to controls expressing the catalytically-inactive mutants PTEN-C124S or PTEN-G129E (Figure 7). Our inability to recover wild type PTEN-positive cells appears to result from the induction of apoptosis in these cell lines, as expression of PTEN increased the number of cells staining positively for Annexin V. Significantly, co-expression of a constitutively active, membrane targeted PKB/Akt (Andjelkovic, M. *et al. J. Biol. Chem. 272*:31515-31524 (1997)) completely reverted the PTEN phenotype, in that PTEN positive/GFP positive cells could now be recovered despite the presence of wild type PTEN. These data demonstrate that PTEN functions as an upstream regulator of PKB/Akt and, at least in this case, serves to regulate survival signals. The ability of PTEN to inhibit downstream targets of PI 3-kinase, as well as the biochemical data demonstrating that PTEN specifically dephosphorylated the 3-position of phosphatidyl inositol phosphate demonstrates that PTEN is a functional antagonist of signaling events induced by PI 3-kinase.

The activation of PI 3-kinase has been shown to be required for tumor cell invasion (Shaw, L.M. *et al. Cell 91*:949-960 (1997)) and both PI 3-kinase and PKB/Akt have been isolated as transforming oncogenes in retroviruses (Bellacosa, A. *et al. Science 254:* 274-277(1991), Chang, H.W. *et al. Science 276*:1848-1850 (1997)). These data indicate that PI 3-kinase and PKB/Akt function in a growth promoting signaling pathway. Our data demonstrates that PTEN functions to suppress these growth promoting signals by dephosphorylating the phospholipid products of PI 3-kinase. Loss of PTEN would result in the accumulation of these phospholipids and lead to the suppression of the apoptotic machinery.

The present invention relates to an isolated nucleic acid sequence encoding a PTEN, which dephosphorylates phosphorylated tyrosine residues, phosphorylated serine residues, phosphorylated threonine residues and combinations thereof, and which possesses substrate specificity such that the phosphatase activity is enhanced by the presence of acidic amino acid residues in a substrate. The invention pertains to an isolated nucleic acid sequence which encodes a dual specificity phosphatase PTEN having an alteration in amino acid sequence such that the phosphatase activity is inhibited, and having homology to tensin. The invention also relates to a nucleic acid sequence that encodes a phosphatase capable of removing phospate from the 3-position of inositol and phosphatidylinositol. The invention further relates to nucleic acid sequences which hybridize to nucleic acid sequence encoding PTEN or altered PTEN. The invention also relates to PTEN or altered PTEN encoded by the isolated nucleic acid sequences described herein.

As used herein, "alteration" is intended to mean any change in nucleic acid sequence that results in, but is not limited to, missense, nonsense, frame shift, addition or deletion mutations or combinations thereof, in the wild type nucleic acid sequence. Alterations in either the nucleotide sequence or the amino acid sequence of the phosphatase include additions, substitutions and deletions of one or more nucleotides or amino acid residues. The terms "dual specificity phosphatase" or "dual specific phosphatase" refer to an enzyme which removes phosphate from phosphorylated tyrosine, phosphorylated serine, phosphorylated threonine and combinations thereof.

The term "isolated" is used herein to indicate that the material in question (e.g., DNA) exists in a physical milieu distinct from that in which it occurs in nature. For example, the isolated protein of the invention may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs. The phrase "isolated gene or nucleotide sequence" also includes a gene or nucleotide sequence which is synthesized chemically or by recombinant means. Thus, recombinant DNA contained in a vector is included in the definition of "isolated", as used herein. Also, isolated nucleotide

sequences include recombinant DNA molecules in heterologous host cells, as well as partially or substantially purified DNA molecules in solution. *In vivo* and *in vitro* RNA transcripts of the DNA molecules of the present invention are also encompassed by "isolated" nucleotide sequences. Such isolated nucleotide sequences are useful in the manufacture of the encoded protein, as probes for isolating homologous sequences (e.g., from other mammalian species), for gene mapping (e.g., by *in situ* hybridization with chromosomes), or for detecting expression of the PTEN or altered PTEN gene in tissue (e.g., human tissue), such as by Northern blot analysis. Methods of isolating nucleic acid, manufacturing the encoded protein, gene mapping and nucleic acid hybridization (such as Northern blot analysis) are well known in the art.

The present invention also pertains to nucleotide sequences which are not necessarily found in nature but encode PTEN or altered PTEN. Thus, DNA molecules which comprise a sequence which is different from the naturally-occurring PTEN-encoding or altered PTEN-encoding nucleotide sequence but which, due to the degeneracy of the genetic code, encode PTEN or altered PTEN of the present invention, are the subject of this invention. The invention also encompasses nucleotide sequences which encode portions, analogues or derivatives of PTEN or altered PTEN. These nucleotide sequences can be naturally-occurring, such as in the case of allelic variation, or non-naturally-occurring, such as those induced by various mutagens and mutagenic processes. Suitable variations include, but are not limited to, addition, deletion and substitution of one or more nucleotides which can result in conservative or non-conservative amino acid changes, including additions and deletions. The nucleotide or amino acid variations can be silent or conserved; such that they do not alter the characteristics or activity of PTEN or altered PTEN.

In the present invention, the term nucleic acid sequence is intended to include both double-stranded and single-stranded RNA and DNA polymers having a length sufficient to hybridize to PTEN or altered forms thereof via the sense or anti-sense strand. Preferably, the nucleic acid molecule comprises at least about 25 nucleotides,

more preferably at least about 50 nucleotides, and even more preferably at least about 200 nucleotides. The nucleotide sequence can be only that which encodes at least a fragment of the amino acid sequence of the PTEN protein or altered PTEN protein; alternatively, the nucleotide sequence can include at least a fragment of the PTEN or altered PTEN amino acid coding sequence along with additional non-coding sequences, such as introns and non-coding 3' and 5' sequences (including regulatory sequences, for example). Additionally, the nucleotide sequence can be fused to a marker sequence, for example, a sequence which encodes a polypeptide to assist in isolation or purification of the polypeptide. Such sequences include, but are not limited to, those which encode a glutathione-S-transferase (GST) fusion protein, a hemaglutin A (HA) peptide marker from influenza, a Myc peptide marker from the myc oncogene and a polyhistidine tag.

In particular embodiments, the altered PTEN phosphatase has an alteration within the catalytic domain. In one embodiment, the alteration is a point mutation, such as a substitution of tyrosine to histidine at amino acid 123 or a substitution of arginine to glycine at amino acid 129. In another embodiment, the encoded PTEN phosphatase has an alteration in alpha helix 2 or in alpha helix 7, wherein alpha helices 2 and 7 are structural motifs defined by the crystal structure of vaccinia H1-related phosphatase (VHR) (Yuvaniyama, J *et al. Science* 272:1328-1331 (1996)). For example, the alteration can be a point mutation, such as a substitution of arginine for serine at amino acid 170, a substitution of arginine for glycine at amino acid 165, and a substitution of proline for threonine at amino acid 167. The altered nucleic acid or amino acid sequences can be obtained by mutagenesis of sequences from naturally-occurring sources, or can be recombinantly produced or synthesized using techniques well known in the art, e.g. by chemical or other methods.

The present invention further pertains to antibodies or antigen-binding fragments thereof which specifically bind to PTEN or altered PTEN as described herein, and to nucleic acid probes which hybridize to nucleic acid sequences encoding the altered PTEN phosphatase as described herein. The invention also relates to use of the

compositions described herein, such as in assays to measure phosphatase activity. Compositions and methods described herein can be used in the diagnosis, therapy or prophylaxis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual. Generally, the method comprises combining a biological sample to be tested (e.g., cells, tissue or bodily fluids) with appropriate reagents for detecting an alteration in PTEN. As used herein, alteration of phosphatase activity is intended to include both inhibition and enhancement of catalytic activity. As used herein, inhibition includes any detectable reduction in the indicated measure of interest, including reduction of activity and complete loss of activity. Enhancement includes any increase in activity. A sample to be tested can be any biological sample, such as blood, lymph, urine, tissue extracts or cellular extracts. A sample of cells can be bodily fluids, tissues, cell culture samples and purified or non-purified protein samples.

In one embodiment, the invention relates to a method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual. In one embodiment of the method, the occurrence (presence or absence and/or quantity) of DNA or RNA encoding PTEN or altered PTEN is detected using hybridization-based techniques. This embodiment of the method comprises the steps of rendering nucleic acids in a sample of cells from the individual available for hybridization with complementary nucleic acids; combining the resulting product with a nucleic acid probe which detects an alteration in nucleic acid sequences encoding an altered form of PTEN which causes an alteration of phosphatase activity of PTEN, under conditions appropriate for hybridization of complementary nucleic acids in the sample of cells with the nucleic acid probe; and detecting hybridization of nucleic acids in the sample of cells with the nucleic acid probe. Appropriate nucleic acid probes are those which hybridize to the altered PTEN sequence and do not hybridize to the wild type PTEN sequence. Hybridization of nucleic acids in the sample of cells with the nucleic acid probes is indicative of a

condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN. In particular embodiments, the nucleic acid probe detects an alteration in the catalytic domain, alpha helix 2 or alpha helix 7 of PTEN. In another embodiment, the condition is tumorigenesis.

For example, nucleic acid probes provided by the present invention can be used to screen by single strand conformation polymorphism (SSCP) (Orita, M. *et al. Proc. Natl. Acad. Sci. USA* 86:2766-2770 (1989), Suzuki, Y., *et al. Oncogene* 5:1037-1043 (1990), Murakami, Y., *et al* Oncogene 6:37-42 (1991)), hybridization, sequencing or PCR for specific mutations which lead to an inhibition of phosphatase activity. These detection methods are well established in the art. The probe can be labeled in a suitable manner for detection of single nucleotide substitution by mobility shift analysis, hybridization between the probe and nucleic acids derived from a suitable sample or for direct sequencing of nucleic acids derived from a suitable sample. Furthermore, PCR products may be detected by standard electrophoresis techniques. These probes can vary in length from 10 nucleotides to more than 200 nucleotides, and will correspond to unique sequences present in PTEN or altered forms thereof.

In another embodiment of the present method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual, the occurrence of PTEN and/or altered PTEN is detected using antibody techniques well known in the art. This embodiment of the method comprises rendering proteins in a sample of cells from the individual available for binding with antibodies, e.g., by disrupting the cells with an appropriate detergent in a suitable buffer solution; combining the resulting product with an antibody that binds altered PTEN, under conditions appropriate for binding of the antibody to proteins; and detecting antibody binding to altered PTEN in the sample. Binding of the antibody to altered PTEN is indicative of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN.

A composition comprising an antibody is intended to include both the antibody itself and source which express the desired antibody, including any source of monoclonal or polyclonal antibodies that interact specifically with PTEN or altered forms thereof. Sources which expresses the desired antibody, include, but are not limited to, serum, ascites fluid, primary culture, hybridomas, and conditioned media derived from primary culture or hybridomas.

Specific antibodies can be used to detect the presence of PTEN or altered forms of the phosphatase with inhibited phosphatase activity, using standard enzyme-linked immunosorbant assay, radioimmunoassay and immunoblot analysis. Specific antibodies of the present invention can also be used for immuno-cytochemistry on cells or tissues. For example, polyclonal and monoclonal antibodies, including non-human and human antibodies, humanized antibodies, chimeric antibodies and antigen-binding fragments thereof (*Current Protocols in Immunology*, John Wiley & Sons, N.Y. (1994); EP Application 173,494 (Morrison); International Patent Application WO86/01533 (Neuberger); and U.S. Patent No. 5,225,539 (Winters)), which bind to the described PTEN or altered PTEN, are within the scope of the invention. A mammal, such as a mouse, rat, hamster or rabbit, can be immunized with an immunogenic form of PTEN or altered PTEN (e.g., PTEN, altered PTEN, an antigenic fragment of PTEN, or an antigenic fragment of altered PTEN comprising the altered portion of the phosphatase, which are capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. The protein or polypeptide can be administered in the presence of an adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibody.

Following immunization, antisera can be obtained, and if desired, polyclonal antibodies can be isolated from the serum. Monoclonal antibodies can also be produced by standard techniques, which are well known in the art (Kohler and Milstein, *Nature*

256:495-497 (1975); Kozbar et al., *Immunology Today 4*:72 (1983); and Cole et al., *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. pp. 77-96 (1985)). The term "antibody" as used herein is intended to include fragments thereof, such as Fab and $F(ab)_2$. Antibodies described herein can be used to inhibit the activity of the phosphatase described herein, particularly *in vitro* and in cell extracts, using methods known in the art. Additionally, such antibodies, in conjunction with a label, such as a radioactive label, can be used to assay for the presence of the expressed protein in a cell from, e.g., a tissue sample, and can be used in an immunoabsorption process, such as an ELISA, to isolate PTEN or altered PTEN. Tissue samples which can be assayed include human tissues, e.g., differentiated and non-differentiated cells. Examples include bone marrow, thymus, kidney, liver, brain, pancreas, fibroblasts and epithelium.

As described herein, a peptide fragment of PTEN, PTEN Glu-388-Val403, was used to generate polyclonal, anti-PTEN antiserum in a rabbit. The resultant antibody is designated PTEN486. Thus, the invention also pertains to an anti-PTEN antibody, particularly PTEN486, which binds PTEN at the C-terminal region. This antibody can be used to identify and/or isolate both wild type PTEN and altered PTEN, provided that the altered PTEN is not altered such that the C-terminal 16 amino acid residues are deleted.

In a further embodiment of the present method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual, occurrence of PTEN or altered PTEN, or DNA or RNA encoding PTEN or altered PTEN is assessed using phosphatase activity assays. Such assays are well known in the art. This embodiment of the method comprises rendering proteins in a sample of cells from the individual available for assessment of phosphatase activity; combining the resulting product with a phosphorylated PTEN substrate, under conditions appropriate for dephosphorylation of the phosphorylated substrate by PTEN; and detecting dephosphorylation of the phosphorylated substrate. In one preferred embodiment, PTEN is isolated (i.e.,

purified) from a crude (non-purified) sample using specific antibody, and utilized in the phosphatase assay as described. In another preferred embodiment, a specific substrate of PTEN is utilized in the phosphatase assay described in conjunction with either purified PTEN or a non-purified sample containing PTEN. Inhibition of dephosphorylation relative to an appropriate control or reference sample is indicative of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN. An appropriate control or reference sample is the amount of dephosphorylation in a cell sample known to have normal (e.g., wild type) PTEN activity. The control or reference sample activity can be determined simultaneously with, prior to or after the assessment of the sample being tested.

The present invention provides a method to be used in the diagnosis of cancers, e.g., brain, prostate and breast cancers and Cowden's disease, or in the determination of pre-disposition thereto. As used herein, "condition" is intended to include active disorders, e.g., disorders which have manifested their symptoms, and predisposition to a disorder (e.g., the genetic tendency toward a disorder which has not yet manifested itself symptomatically). This invention also provides a method to diagnose conditions involving altered forms of PTEN, for example using phosphatase assays which combine suitable substrates, such as PtdIns(3,4,5)P_3 or phosphorylated polyGlu$_4$Tyr$_1$, with tissue, cellular, or protein samples in an appropriate assay as described herein, wherein inhibition of phosphatase activity is indicative of an alteration in PTEN.

Furthermore, the present invention may be used in the treatment of conditions in which enhancement or inhibition of PTEN phosphatase activity is desirable. In one embodiment, the cells are contacted with a nucleic acid molecule encoding PTEN on an altered form thereof. As used herein, contacting means adding the PTEN expressing construct to the extracellular space of the cells, transfecting the cells with a PTEN expressing nucleic acid construct or targeting said construct to specific cell types, such as PTEN deficient cells. Methods of adding, transfecting and targeting are well known in the art. For example, wild type PTEN or a nucleic acid encoding wild type PTEN, or

a mimic thereof, may be administered in an appropriate vehicle, with an optional physiological composition to an individual having a condition characterized by lack of PTEN phosphatase activity (leading to a condition such as a hyper-proliferative condition). The condition is alleviated due to the activity of the introduced PTEN. The hyperproliferative condition may include brain, prostate or breast cancers in addition to Cowden's disease and other hyperproliferative diseases involving reduced PTEN phosphatase activity.

In addition, altered forms of PTEN, or a nucleic acid sequence encoding altered forms of PTEN may be administered in an appropriate vehicle with an optional physiological composition to an individual in whom an increase in PTEN phosphatase activity is desirable, such as, for example, to induce apoptosis in proliferative or hyperproliferative cells. Methods of measuring apoptosis are well known in the art. In addition, altered forms of PTEN, or a nucleic acid sequence encoding altered forms of PTEN may be administered in an appropriate vehicle with an optional physiological composition to an individual in whom reduction or attenuation of PTEN phosphatase activity is desirable. Administration alleviates the condition, which can include disorders such as degenerative conditions. The altered forms of PTEN or nucleic acids encoding the altered forms of PTEN may derived from the altered forms described herein, including a "substrate trapping" form of PTEN (as described in U.S. patent application Serial No. 08/685,992, filed July 25, 1996). In such altered forms, the altered PTEN is active in cells whereby the endogenous phosphatase and the introduced, substrate trapping PTEN share a common substrate, such that the overall activity of the phosphatase is reduced due to the sequestration of the substrate. The degenerative condition may include Parkinson's and other neurodegenerative diseases which may involve PTEN phosphatase activity.

The present invention also pertains to pharmaceutical compositions comprising polypeptides described herein. For instance, a polypeptide or protein, or product thereof, of the present invention can be formulated with a physiologically acceptable

medium to prepare a pharmaceutical composition. The particular physiological medium may include, but is not limited to, water, buffered saline, polyols (e.g., glycerol, propylene glycol, liquid polyethylene glycol) and dextrose solutions. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to well known procedures, and will depend on the ultimate pharmaceutical formulation desired. Methods of introduction of exogenous peptides at the site of treatment include, but are not limited to, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, oral and intranasal. Other suitable methods of introduction can also include gene therapy, rechargeable or biodegradable devices and slow release polymeric devices. The present invention is related to use of PTEN or a nucleic acid molecule encoding PTEN, for the manufacture of a medicament for use in a method of treating a condition in an individual in which enhancement of PTEN phosphatase activity is desirable. The present invention is also related to use of an altered form of PTEN or a nucleic acid molecule encoding an altered form of PTEN, for the manufacture of a medicament for use in a method of treating a condition in an individual in which reduction of PTEN phosphatase activity is desirable. The present invention is further related to use of PTEN or a nucleic acid molecule encoding PTEN, for the manufacture of a medicament for use in a method of treating prostate cancer. The pharmaceutical compositions of this invention can also be administered as part of a combinatorial therapy with other agents.

The following Examples are offered for the purpose of illustrating the present invention and are not to be construed to limit the scope of this invention. The teachings of all references cited herein are hereby incorporated herein by reference.

EXAMPLES

Example 1: Isolation and Purification of PTEN.

A full length PTEN cDNA was generated by ligating the Not1-Bg1II fragment from EST264611 with the Bg1II-EcoR1 fragment of EST365465 into pBluescript digested with Not1-EcoRI. The resulting full length PTEN cDNA was amplified by PCR using pfu polymerase (Stratagene) and primers that add a 5' BamHI site (5'CGCGGATCCATGACAGCCATCATCAAAGAGATCGTTAGC) (SEQ ID NO: 3) and a 3' EcoRI site (5'CGCGAATTCTCAGACTTTTGTAATTTGTGTATGC) (SEQ ID NO: 4). The resulting fragment was subcloned into pGEX2T (Pharmacia) and the sequence verified by automated sequencing. Expression of PTEN was induced in 500 ml of mid-log phase bacteria (A_{600}=0.600) by the addition of IPTG to 200 μM. The culture was shifted to room temperature and expression was allowed to proceed for 12 hours. The bacteria were harvested by centrifugation, the supernatant was removed and the bacterial pellet frozen at -80°C. The frozen pellets were resuspended in 5 ml of ice cold 20 mM Tris, 150 mM NaCl and 5 mM EDTA, pH 8.0 supplemented with lysozyme (1 mg/ml), aprotinin (μg/ml), leupeptin (5 μg/ml), and benzamidine (1 mM) and incubated on ice for 15 minutes. The bacteria were lysed by sonicating 3 times for 1 minute each with a Branson Model 450 sonifier, power setting 4, 70% duty cycle. The lysate was cleared by centrifugation at 30,000 x g for 10 minutes, diluted with an equal volume of HBS (50 mM Hepes, 150 mM NaCl, pH 7.4). Glutathione-Sepharose 4B (300 μL) was added, and the resulting slurry was incubated at 4°C on a rocking platform for 1-2 hours. The glutathione-Sepharose was washed 5 times, each with 10 ml of ice cold HBS and the washed fusion proteins were eluted with a solution containing 20 mM glutathione, 50 mM Hepes and 30% glycerol, pH 8.0. Protein concentrations were determined by the method of Bradford, using BSA as a standard, and the integrity of the fusion proteins were verified by SDS-polyacrylamide gel electrophoresis. Mutations identified from tumor samples and cell lines (4,5 and Dr. L. Hedrick (Johns Hopkins University) personal communication) were introduced into pGEX2T-PTEN using the Quickchange mutagenesis kit as described by the manufacturer (Stratagene). For all mutations, the entire PTEN open reading frame was

sequenced to confirm that no other mutations had been introduced. The DNA sequence and translation are shown in Figures 1A-1C.

Example 2: Characterization of PTEN

Preparation of substrates

All tyrosine phosphorylated substrates were phosphorylated with the cytoplasmic fragment of the β subunit of the insulin receptor kinase (βIRK) and purified as described (Flint, A.J. *et al. Embo J.* 12:1937-1946 (1993)). Serine phosphorylated substrates were phosphorylated with recombinant protein kinase A (New England Biolabs) or with recombinant casein kinase II (a gift from Dr. D. Litchfield, University of Western Ontario) in a reaction mixture consisting of 50 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 2mM DTT, 2 mM ATP, and 1 mCi of [γ-^{32}P] ATP in a total volume of 1 ml. Casein was used at a concentration of 10 mg/ml, MBP at 4 mg/ml and the peptides RRRDDDSDDD (SEQ ID NO: 5) (DSD) and RRREEETEEE (SEQ ID NO: 6) (ETE), were used at a concentration of 0.5 mg/ml. Protein substrates were precipitated by the addition of ammonium sulfate to 80%, incubated on ice for 30 minutes and harvested by centrifugation. the precipitated proteins were washed 3 times with 80% ammonium sulfate and then resuspended in 500 µl of 1 M Hepes, pH 7.5. The solubilized proteins were dialyzed against several changes of 50 mM Imidizole, pH 7.2. Peptide substrates were purified using a Sep-pak C18 reverse phase cartridge (Waters) as described (Myers, MP *et al. Mol. Cell. Biol.* 14:6954-6961 (1994)). The purified peptides were lyophilized to dryness and resuspended in 50 mM Imidizole, pH 7.2. Random copolymers of glutamate and tyrosine, with a 4:1 ratio of glutamate to tyrosine ($polyGlu_4Tyr_1$) or with a 1:1 ratio ($polyGlu_1Tyr_1$), were purchased from Sigma and phosphorylated with βIRK at a final polymer concentration of 1 mg/ml and purified, as described, using Sep-pak C18 reverse phase chromatography. Phosphorylated ERK2 was produced in *E. coli* by co-expression of activated MEK and was a gift from Dr. D. Barford (Oxford University).

Phosphatase Assays

The standard phosphatase assay contained 10 μM substrate, 50 mM Hepes, pH 7.0, 10 mM $MgCl_2$, 10 mM DTT. The reaction was initiated by the addition of enzyme, typically 1-2 μg, to prewarmed (30°C) substrate mix, resulting in a final volume of 60 μl. The reactions were allowed to proceed at 30°C for the indicated times and stopped by the addition of a suspension of activated charcoal in 900 mM HCl, 90 mM NaPPi and 2 mM NaPi (Zhang, S.-H., *et al. J. Biol. Chem.* 270:20067-20072 (1995)).

Substrate Specificity of Purified PTEN

Initially, as described herein, PTEN was assayed against a number of tyrosine phosphorylated proteins and peptides, including reduced carboxyamidomethylated and maleylated lysozyme (RCML), mylelin basic protein (MBP), $polyGlu_4Tyr_1$ and $polyGlu_1Tyr_1$, as well as the peptide EDNDYINASL. The activity of PTEN towards the classical substrates RCML and MBP was weak (<80 pmol/min/mg). However, PTEN, exhibited robust phosphatase activity when $polyGlu_4Tyr_1$ was used as a substrate (4840 pmol/min/mg) (Table and Figure 2A). Significantly, addition of unphosphorylated $polyGlu_4Tyr_1$ to reactions using RCML as a substrate did not result in an increase in activity towards RCML, indicating that PTEN was not activated by the polyanionic character of $polyGlu_4Tyr_1$. In fact, inclusion of unphosphorylated $polyGlu_4Tyr_1$ in reactions containing RCML resulted in the inhibition of the already limited dephosphorylation of RCML, suggesting that even unphosphorylated $polyGlu_4Tyr_1$ was capable of binding to PTEN, displacing the more weakly interacting RCML. As described herein, PTEN also dephosphorylated $polyGlu_1Tyr_1$. When the stoichiometries of phosphorylation were normalized, no significant differences were detected in the rates of dephosphorylation of $polyGlu_4Tyr_1$ and $polyGlu_1Tyr_1$, suggesting that the acidic character of these substrates is an important determinant of substrate recognition.

PTEN, however, exhibited reduced specific activity when assayed with the acidic peptide EDNDYINASL (Table), suggesting that the mere presence of acidic residues was not sufficient to ensure that a peptide would be a substrate. Cdc14, a dual specificity phosphatase that is closely related to PTEN (Liaw, D. *et al., Nature Genet.* 16:64-67 (1997)), does not discriminate between RCML and $polyGlu_4Tyr_1$ (Figure 2C), indicating that $polyGlu_4Tyr_1$ is not a universal substrate for the dual specificity phosphatases, and that the substrate specificity of PTEN is likely to be unique feature determined by structural motifs separate from the catalytic motif.

Dual Specificity Phosphatase Activity of PTEN

As described herein, in order to test whether PTEN falls into the class of dual specificity phosphatases, PTEN activity was assayed using a number of proteins and peptides phosphorylated on serine and threonine residues. Similar to the findings with tyrosine phosphorylated substrates, PTEN dephosphorylated serine/threonine residues in substrates that had a preponderance of acidic residues. Specifically, PTEN dephosphorylated two peptide substrates (DSD and ETE) with the highest efficiency (Table and Figure 2B). Furthermore, PTEN showed specificity even amongst acidic serine/threonine substrates, exhibiting a reduced activity when casein, phosphorylated by casein kinase II or protein kinase A, was used as substrate (Table and Figure 2B). As might be anticipated in light of these properties, PTEN exhibited almost undetectable activity when assayed with polybasic substrates, such as MBP or Kemptide (LRRASLG). The finding that PTEN dephosphorylated two peptide substrates of casein kinase II also indicates that the inability of PTEN to dephosphorylate EDNDYINASL does not simply reflect its inability to dephosphorylate small peptide substrates. Although many dual specificity phosphatases show a preference for tyrosine residues, it is possible that the reduction in the specific activity of PTEN towards serine/threonine substrates may be the result of these residues being located in a suboptimal substrate backbone.

Generation of PTEN Antibodies

The following PTEN peptides were synthesized by the Cold Spring Harbor Laboratory core facility: PTEN Glu388-Val403 (Cys-Glu-Asn-Glu-Pro-Phe-Asp-Glu-Asp-Gln-His-Thr-Gln-Thr-Lys-Val), PTEN Lys6-Asp19 (Lys-Glu-Ile-Val-Ser-Arg-Asn-Lys-Arg-Arg-Tyr-Gln-Glu-Asp-Cys), PTEN Scr226-Lys237 (Cys-Ser-Ser-Asn-Ser-Gly-Pro-Thr-Arg-Arg-Glu-Asp-Lys), PTEN Thr321-Lys332 (Cys-Thr-Lys-Asn-Asp-Leu-Asp-Lys-Ala-Asn-Lys-Asp-Lys). Underlined residues are not found in corresponding peptides of wild type PTEN and were added to aid in the conjugation of the peptides to Keyhole Limpet Hemocyanin (KLH). Female New Zealand White rabbits were immunized with recombinant PTEN produced as a GST-fusion protein in bacteria, as well as the KLH-peptide conjugates utilizing standard protocols. Bleeds were initially tested for the ability of the antibodies to immunoblot recombinant PTEN produced in bacteria and were then tested for their ability to immunoblot and immunoprecipitate endogenously expressed PTEN. Rabbit 486, which was injected with PTEN Glu388-Val403 produced antibodies that recognized endogenous PTEN on immunoblots and in immunoprecipitations (this antibody will be referred to as PTEN486). All other rabbits failed to produce antibodies that could recognize PTEN on immunoblots or in immunoprecipitations.

Example 3: Dephosphorylation of ERK2

Dephosphorylation of ERK2 was performed essentially as described for the radioactive substrates; at the indicated times duplicate aliquots were removed and stopped by the addition of 5X Laemmli sample buffer and processed for immunoblot analysis as described (Myers, MP *et al. Mol. Cell. Biol.* 14:6954-6961 (1994)). Immuno-blots were probed with a 1:5000 dilution of anti-MAP kinase ascites or with a mixture of anti-phosphotyrosine antibodies (1:2000 dilution of G-104 and G-2-98 ascites (Garton, A.J. *et al. Mol. Cell. Biol. 16*:6408-6418 (1996)) and were developed with ECL reagents (Amersham) in conjunction with an HRP-labeled rat anti-mouse

Kappa chain antibody (Zymed). A number of dual specificity phosphatases show a preference for members of the MAP kinase family (Sun, H., *et al. Cell.* 75:487-493 (1993), Kwak, S.P. *et al. J. Biol. Chem.* 269:3569-3604 (1994), Mourey, R.J., *et al. J. Cell Biol.* 271:3795-3802 (1996)). Therefore, PTEN was tested for its ability to dephosphorylate ERK2. As described herein, dephosphorylation of ERK2 by PTEN could not be detected, either by changes in antiphosphotyrosine antibody reactivity or by changes in the electrophoretic mobility of ERK2. In contrast, MKP-1 quickly and completely dephosphorylated ERK2.

Example 4: Tumor-Derived Point Mutations

As described herein, in order to test whether the activity of PTEN was altered during tumorigenesis, the effect on activity of a variety of point mutations that occur in PTEN isolated from tumor specimens was assessed. Many of these mutations occur in or near the catalytic motif of PTEN including: histidine 123 mutated to tyrosine (H123Y), glycine 129 mutated to arginine (G129R) and methionine 134 mutated to leucine (M134L). In addition, several mutations were found that occurred outside the conserved catalytic motif including a mutation in leucine 57 (L57W) and a cluster of mutations C-terminal to the catalytic loop (glycine 165 to arginine (G165R), threonine 167 to proline (T167P) and serine 170 to arginine (S170R)). The exact positions of these mutations are indicated in Figure 3.

Example 5: Tumor-Derived Point Mutations Cause Inactivation of PTEN Phosphatase Activity

GST fusion proteins were produced as described in Example 1, in which PTEN was mutated at each of these positions, to mimic the mutant alleles. The phosphatase activity of the resulting recombinant proteins was measured using $polyGlu_4Tyr_1$ as a substrate (Figure 4). With the notable exception of the M134L mutation, all the mutations tested resulted in a dramatic decrease in the activity of PTEN (Figure 4).

These data indicate that the catalytic activity of PTEN has been disrupted in these tumors. The essentially wild type activity exhibited by the M134L mutation is not unexpected, because many other dual specificity phosphatases contain a leucine residue at this position (Barford, D. *Curr. Opin. Struct. Biol.* 5:728-734 (1995)).

Example 6: PTEN Dephosphorylates PtdIns(3,4,5)P_3

Phosphatase Assays:

Recombinant wild type and mutant forms of PTEN were expressed in *E. coli* and assayed with polyGlyTyr as shown in Edxmaple 2 and described (Myers, M.P. *et al. Proc. Natl. Acad. Sci. USA 94*:9052-9057 (1997)). Release of $^{32}P_i$ from radiolabeled phosphatidyl inositol phosphate was determined by performing a modified Bligh and Dyer extraction on the reaction mix (Bligh, E.J. and Dyer, W.J. *Can. J. Biochem. Physiol. 37*:911-916 (1959)). The pooled upper phases (containing inorganic phosphate) were removed, dried down and resuspended in a 1M TCA, 1% ammonium molybdate solution. Following extraction with 2 volumes of toluene:isobutylalchohol (1:1) the upper phase was removed and counted. Site selectively was determined by incubating recombinant PTEN or SHIP (a gift from C. Erneux, Free University, Brussels) with radiolabeled PtdIns(3,4,5)P_3 and the lipid products from these reactions were analyzed by thin layer chromatography or HPLC. Briefly, the phospholipids were extracted by performing a Bligh and Dyer extraction and the pooled lower phases were dried down and resuspended in 20 μ1 of chloroform:methanol (2:1) and applied to an oxalate activated silica gel TLC plate. Plates were developed in methanol/chloroform/ water/ammonia (100:75:25:15) and the phospholipids were detected by autoradiography. PtdIns(3,4,5)P_3 mass assays were performed as described (van der Kaay, J. *et al. J. Biol. Chem. 272:* 5477-5481 (1997)).

Figure 5 demonstrates that PTEN dephosphorylates PtdIns(3,4,5)P_3. In panel A, recombinant PTEN was incubated with radiolabeled polyGluTyr and PtdIns(3,4,5)P_3 and the release of $^{32}P_i$ was measured as described. In panel B, PtdIns(3,4,5)P_3,

PtdIns(3,4,)P_2, or PtdIns(3)P were incubated with PTEN and the release of $^{32}P_i$ was determined as described.

Example 7: Expression of PTEN Antagonizes PI-3 Kinase

Tissue Culture and Transfection:

All cell lines were maintained in Dulbecco's Modified Eagle's Medium supplemental with 10% fetal calf serum and antibiotics. HEK293 cells were transfected via calcium phosphate co-precipitation with 20 μg of DNA per 10 cm dish. The calcium phosphate:DNA co-precipitate was removed by washing with PBS 16 hours after addition and the cells were returned to growth medium for 36 hours before harvesting. Co-transfection of PTEN and p110 constructs were performed using 9.5 μg of each plasmid DNA. Transfection efficiency was determined by including a GFP expression plasmid (1 μg) in all transfections.

Figure 6 demonstrates that expression of PTEN antagonizes PI 3-kinase. HEK293 cells were transfected with PTEN or PTENC124S in combination with p110 PI 3-kinase or an activated, membrane bound PI-3 kinase (p110-CAAX). The resulting levels of PtdIns(3,4,5)P_3 were determined as described and were normalized to total protein. Data is expressed as pmol PtdIns(3,4,5)P_3 per mg protein.

Example 8: Expression of PTEN in Glioblastoma Cell Lines Decreases the Amount of Activated PKB/Akt

PTEN retroviral expression vectors were constructed in pBabePuro. Following transfection into packaging lines, the viral supernatants were harvested, diluted with growth media (described above) and incubated with U87 MG or U373 MG cells for 16 hours at 30° C. Infected cells were selected with puromycin (1.5 μg/ml) and drug resistant colonies were expanded to generate clonal cell lines.

Cells lysates and immunoblots were performed essentially as described (Zhang, S.H. *et al. J. Biol. Chem. 272*:27281-27287 (1997)). Protein levels were determined by the method of Bradford using BSA as a standard and equal protein was loaded in each

linc. Antibodies to PTEN were generated in rabbits using a C-terminal peptide of PTEN conjugated to KLH. Antibodies to PKB/Akt, phospho-PKB/Akt (specific to phosphorylated Ser473) and phospho-BAD were purchased from New England Biolabs.

Expression of PTEN in glioblastoma cell lines decreases the amount of activated PKB/AKT. PTEN expression was reconstituted in glioblastoma cell lines U87 MG, or U373 MB, by infecting with recombinant retrovirus. Confluent dishes were either left untreated, stimulated with insulin (10μg/ml for 10 minutes), stimulated with PDGF (50 ng/ml for 10 minutes) or pretreated with Wormtannin (150 nM for 30 minutes) and then stimulated with both insulin (10μg/ml) and PDGF (50 ng/ml) for 10 minutes and then lysed.

Example 9: Expression of PTEN Induces Apoptosis in LnCaP Cells

LnCaP cells, a PTEN deficient prostate cancer cell line, were transfected using cationic lipids (Transfast, Promega) at a DNA:lipid ratio of 1. Cells were incubated with the DNA:lipid complexes for 12 hours and then transfected cells were identified based on the expression of a co-transfected GFP vector or by performing immunofluorescence using antibodies to the HA-epitope tag located at the N-terminus of PTEN essentially as described (Tiganis, T. *et al. Mol. Cell Biol. 18*:1622-1634 (1998)). Apoptotic cells were detected by staining with Annexin V (Clontech). Cells were washed with binding buffer (20 mM Hepes, pH 7.4, 137 mM NaCl, 2 mM $CaCl_2$) and then incubated for 30 minutes with Annexin V (500 ng/ml) in the same buffer. Cells were washed twice with binding buffer and then fixed with 4% paraformaldehyde (in binding buffer) for 30 minutes at 4° C and then processed for immunofluorescence.

Figure 7 demonstrates that expression of PTEN induces apoptosis in LnCaP cells. PTEN, PTENC124S, or PTENG129E, were co-transfected with a green flourescent protein (GFP) expression vector into LnCaP cells. The co-transfections also included either empty vector or a constitutively active PKB/Akt expression vector. Transfected cells were identified as GFP positive cells or by immunofluorescence microscopy following staining with antibodies to the transfected PTEN. As shown in

Figure 7, transfection efficiency was assessed by determining the percentage of GFP/PTEN positive cells (total number of cells was determined by counting nuclei (stained with DAPI). Data are expressed as the mean transfection efficiency (+/- s.d. n=3).

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. Use of P-TEN or a nucleic acid molecule encoding P-TEN, for the manufacture of a medicament for use in a method of treating a condition in an individual in which enhancement of P-TEN phosphatase activity is desirable.

2. Use of an altered form of P-TEN or a nucleic acid molecule encoding an altered form of P-TEN, for the manufacture of a medicament for use in a method of treating a condition in an individual in which reduction of P-TEN phosphatase activity is desirable.

3. Use of P-TEN or a nucleic acid molecule encoding P-TEN, for the manufacture of a medicament for use in a method of treating prostate cancer.

4. An isolated nucleic acid molecule encoding PTEN phosphatase, wherein the encoded PTEN phosphatase dephosphorylates phosphorylated tyrosine residues, phosphorylated serine residues, phosphorylated threonine residues and combinations thereof.

5. An isolated nucleic acid molecule encoding PTEN phosphatase, wherein the encoded PTEN phosphatase removes 3-position phosphate from a phosphoinositide substrate.

6. A phosphatase according to Claim 5, wherein the substrate is selected from the group consisting of: PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, PtdIns3P and Ins(1,3,4,5)P_4.

7. An isolated nucleic acid molecule encoding PTEN phosphatase, wherein the encoded PTEN phosphatase possesses substrate sensitivity such that phosphatase activity is enhanced by presence of acidic amino acid residues in a substrate of the PTEN phosphatase.

8. An isolated PTEN phosphatase, wherein the phosphatase dephosphorylates phosphorylated tyrosine residues, phosphorylated serine residues, phosphorylated threonine residues and combinations thereof.

9. An isolated PTEN phosphatase according to Claim 8, wherein the phosphatase possesses substrate sensitivity such that phosphatase activity is enhanced by presence of acidic amino acid residues in a substrate.

10. An isolated phosphatase, wherein the phosphatase removes 3-position phosphate from a phosphoinositide substrate.

11. A phosphatase according to Claim 10, wherein the phosphatase is PTEN.

12. A phosphatase according to Claim 10, wherein the substrate is selected from the group consisting of: PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, PtdIns3P and Ins(1,3,4,5)P_4.

13. An isolated nucleic acid molecule encoding an altered dual specificity phosphatase, wherein the dual specificity phosphatase has an alteration in amino acid sequence such that phosphatase activity is inhibited.

14. An isolated nucleic acid molecule according to Claim 13 wherein the encoded dual specificity phosphatase is PTEN.

15. An isolated nucleic acid molecule according to Claim 13 wherein the alteration in amino acid sequence is in the catalytic domain of the phosphatase.

16. An isolated nucleic acid molecule according to Claim 15 wherein the alteration is a point mutation.

17. An isolated nucleic acid molecule according to Claim 16 wherein the point mutation is selected from the group consisting of: mutation of histidine 123 to tyrosine, mutation of glycine 129 to arginine and glycine 129 to glutamic acid.

18. An isolated nucleic acid molecule according to Claim 13 wherein the alteration in amino acid sequence occurs outside of the catalytic domain.

19. An isolated nucleic acid molecule according to Claim 18 wherein the alteration occurs within alpha helix 2 or alpha helix 7 of the phosphatase.

20. An isolated nucleic acid molecule according to Claim 18 wherein the alteration is a point mutation.

21. An isolated nucleic acid molecule according to Claim 20 wherein the point mutation is selected from the group consisting of: mutation of serine 170 to arginine; mutation of glycine 165 to arginine; and mutation of threonine 167 to proline.

22. An isolated altered dual specificity phosphatase having an alteration in amino acid sequence such that phosphatase activity is inhibited.

23. An isolated phosphatase according to Claim 22 wherein the phosphatase is PTEN.

24. An antibody that specifically binds to a phosphatase selected from the group consisting of:

a) a phosphatase which dephosphorylates phosphorylated tyrosine residues, phosphorylated serine residues, phosphorylated threonine residues and combinations thereof;

b) a phosphatase which possesses substrate sensitivity such that phosphatase activity is enhanced by presence of acidic amino acid residues in a substrate;

c) an altered dual specificity phosphatase having an alteration in amino acid sequence such that phosphatase activity is inhibited.

25. A method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual, comprising the steps of:

a) rendering nucleic acids in a sample of cells from the individual available for hybridization with complementary nucleic acids;

b) combining the product of a) with a nucleic acid probe which hybridizes with nucleic acids encoding an alteration in PTEN which causes an alteration of phosphatase activity of PTEN, under conditions appropriate for hybridization of nucleic acids in the sample of cells with the nucleic acid probe; and

c) detecting hybridization of nucleic acids in the sample of cells with the nucleic acid probe,

wherein hybridization of nucleic acids in the sample of cells with the nucleic acid probes is indicative of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN.

26. A method of Claim 25 wherein the nucleic acid probe detects an alteration in the catalytic domain, alpha helix 2 or alpha helix 7 of PTEN.

27. A method of Claim 25, wherein the condition is tumorigenesis.

28. A method of Claim 25, wherein the alteration of phosphatase activity is inhibition of phosphatase activity.

29. A method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual, comprising:

a) rendering proteins in a sample of cells from the individual available for binding with antibodies;

b) combining the product of a) with a composition comprising specific antibody which binds altered PTEN having altered phosphatase activity, under conditions appropriate for binding of the antibody to the proteins;

c) detecting antibody binding to altered PTEN in the sample of cells, wherein binding of the antibody to altered PTEN is indicative of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN.

30. A method of Claim 29 wherein the specific antibody detects an altered PTEN selected from the group consisting of: PTEN having a deletion of the

carboxy terminus, PTEN having an altered catalytic domain, PTEN having an altered alpha helix 2 and PTEN having an altered alpha helix 7.

31. A method of Claim 29 wherein the condition is tumorigenesis.

32. A method of Claim 29 wherein the alteration of phosphatase activity is inhibition of phosphatase activity.

33. A method of diagnosing or aiding in the diagnosis of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN in an individual, comprising the steps of:

a) rendering proteins in a sample of cells from the individual available for binding with antibodies;

b) combining the product of a) with a composition comprising specific antibody which binds PTEN or altered PTEN having altered phosphatase activity, under conditions appropriate for binding of the antibody to the proteins;

c) combining the product of b) with a phosphorylated PTEN substrate, under conditions appropriate for dephosphorylation of the phosphorylated substrate by PTEN;

d) detecting dephosphorylation of the phosphorylated PTEN substrate, wherein inhibition of dephosphorylation is indicative of a condition characterized by an alteration in PTEN which causes an alteration of phosphatase activity of PTEN.

34. A method of Claim 33, wherein the phosphorylated substrate is composed of acidic amino acids.

35. A method of Claim 34, wherein the phosphorylated substrate is selected from the list consisting of: PtdIns(3,4,5)P_3, PtdIns(3,4)P_2, PtdIns3P and Ins(1,3,4,5)P_4.

36. A method of treating a condition in an individual in which enhancement of PTEN phosphatase activity is desirable, comprising administering PTEN or a nucleic acid molecule encoding PTEN in an appropriate physiologically acceptable vehicle to the individual.

37. A method of Claim 36, wherein the condition is selected from the group consisting of hyperproliferative diseases and Cowden's disease.

38. A method of Claim 36, wherein the condition is prostate cancer.

39. A method of treating a condition in an individual in which reduction of PTEN phosphatase activity is desirable, comprising administering an altered form of PTEN or a nucleic acid molecule encoding an altered form of PTEN in an appropriate physiologically acceptable vehicle to the individual.

40. A method of Claim 39, wherein the condition is selected from the group consisting of degenerative diseases neurodegenerative diseases.

41. A nucleic acid probe which specifically hybridizes to DNA encoding an altered PTEN or to a complement thereof.

42. An antibody which specifically binds PTEN at the C-terminal region.

43. An antibody of Claim 38 which is PTEN486.

44. A method of treating prostate cancer in a vertebrate comprising administering to a vertebrate in need thereof a composition comprising PTEN or a nucleic acid molecule encoding PTEN.

45. A method of inducing apoptosis in cells comprising increasing the level of PTEN activity in the cells.

46. A method according to Claim 45, wherein the cells are tumor cells.

47. A method according to Claim 46, wherein the tumor cells are prostate tumor cells.

48. A method according to Claim 45 wherein the cells are contacted with a nucleic acid molecule encoding PTEN, or an altered form thereof.

49. A method of reducing the size of a tumor comprising increasing the level of PTEN activity in the cells.

50. A method according to Claim 49, wherein the tumor is a prostate tumor.

51. A method according to Claim 49, wherein the cells are contacted with a nucleic acid molecule encoding PTEN, or an altered form thereof.

Sequence Range: 1 to 1784

```
           9          18          27          36          45
GCG GCC GCG GCG GCT GCA GCT CCA GGG AGG GGG TCT GAG TCG CCT GTC ACC
 A   A   A   A   A   A   A   P   G   R   G   S   E   S   P   V   T>

 54          63          72          81          90          99
ATT TCC AGG GCT GGG AAC GCC GGA GAG TTG GTC TCT CCC CTT CTA CTG CCT
 I   S   R   A   G   N   A   G   E   L   V   S   P   L   L   L   P>

    108         117         126         135         144         153
CCA ACA CGG CGG CGG CGG CGG CGG CAC ATC CAG GGA CCC GGG CCG GTT TTA
 P   T   R   R   R   R   R   R   H   I   Q   G   P   G   P   V   L>

        162         171         180         189         198
AAC CTC CCG TCC GCC GCC GCC GCA CCC CCC GTG GCC CGG GCT CCG GAG GCC
 N   L   P   S   A   A   A   A   P   P   V   A   R   A   P   E   A>

207         216         225         234         243         252
GCC GGC GGA GGC AGC CGT TCG GAG GAT TAT TCG TCT TCT CCC CAT TCC GCT
 A   G   G   G   S   R   S   E   D   Y   S   S   S   P   H   S   A>

    261         270         279         288         297         306
GCC GCC GCT GCC AGG CCT CTG GCT GCT GAG GAG AAG CAG GCC CAG TCG CTG
 A   A   A   A   R   P   L   A   A   E   E   K   Q   A   Q   S   L>

        315         324         333         342         351
CAA CCA TCC AGC AGC CGC CGC AGC AGC CAT TAC CCG GCT GCG GTC CAG AGC
 Q   P   S   S   S   R   R   S   S   H   Y   P   A   A   V   Q   S>

360         369         378         387         396         405
CAA GCG GCG GCA GAG CGA AGG GCA TCA GCT ACC GCC AAG TCC AGA GCC ATT
 Q   A   A   A   E   R   R   A   S   A   T   A   K   S   R   A   I>

    414         423         432         441         450         459
TCC ATC CTG CAG AAG AAA CCC CGC CAC CAG CAG CTT CTG CCA TCT CTC TCC
 S   I   L   Q   K   K   P   R   H   Q   Q   L   L   P   S   L   S>

        468         477         486         495         504
TCC TTT TTC TTC AGC CAC AGG CTC CCA GAC ATG ACA GCC ATC ATC AAA GAG
 S   F   F   F   S   H   R   L   P   D   M   T   A   I   I   K   E>

513         522         531         540         549         558
ATC GTT AGC AGA AAC AAA AGG AGA TAT CAA GAG GAT GGA TTC GAC TTA GAC
 I   V   S   R   N   K   R   R   Y   Q   E   D   G   F   D   L   D>

    567         576         585         594         603         612
TTG ACC TAT ATT TAT CCA AAC ATT ATT GCT ATG GGA TTT CCT GCA GAA AGA
 L   T   Y   I   Y   P   N   I   I   A   M   G   F   P   A   E   R>

        621         630         639         648         657
CTT GAA GGC GTA TAC AGG AAC AAT ATT GAT GAT GTA GTA AGG TTT TTG GAT
 L   E   G   V   Y   R   N   N   I   D   D   V   V   R   F   L   D>
```

Figure 1A

```
666         675         684         693         702         711
TCA AAG CAT AAA AAC CAT TAC AAG ATA TAC AAT CTT TGT GCT GAA AGA CAT
 S   K   H   K   N   H   Y   K   I   Y   N   L   C   A   E   R   H>

    720         729         738         747         756         765
TAT GAC ACC GCC AAA TTT AAT TGC AGA GTT GCA CAA TAT CCT TTT GAA GAC
 Y   D   T   A   K   F   N   C   R   V   A   Q   Y   P   F   E   D>

        774         783         792         801         810
CAT AAC CCA CCA CAG CTA GAA CTT ATC AAA CCC TTT TGT GAA GAT CTT GAC
 H   N   P   P   Q   L   E   L   I   K   P   F   C   E   D   L   D>

819         828         837         846         855         864
CAA TGG CTA AGT GAA GAT GAC AAT CAT GTT GCA GCA ATT CAC TGT AAA GCT
 Q   W   L   S   E   D   D   N   H   V   A   A   I   H   C   K   A>

    873         882         891         900         909         918
GGA AAG GGA CGA ACT GGT GTA ATG ATA TGT GCA TAT TTA TTA CAT CGG GGC
 G   K   G   R   T   G   V   M   I   C   A   Y   L   L   H   R   G>

        927         936         945         954         963
AAA TTT TTA AAG GCA CAA GAG GCC CTA GAT TTC TAT GGG GAA GTA AGG ACC
 K   F   L   K   A   Q   E   A   L   D   F   Y   G   E   V   R   T>

972         981         990         999        1008        1017
AGA GAC AAA AAG GGA GTA ACT ATT CCC AGT CAG AGG CGC TAT GTG TAT TAT
 R   D   K   K   G   V   T   I   P   S   Q   R   R   Y   V   Y   Y>

   1026        1035        1044        1053        1062        1071
TAT AGC TAC CTG TTA AAG AAT CAT CTG GAT TAT AGA CCA GTG GCA CTG TTG
 Y   S   Y   L   L   K   N   H   L   D   Y   R   P   V   A   L   L>

       1080        1089        1098        1107        1116
TTT CAC AAG ATG ATG TTT GAA ACT ATT CCA ATG TTC AGT GGC GGA ACT TGC
 F   H   K   M   M   F   E   T   I   P   M   F   S   G   G   T   C>

1125        1134        1143        1152        1161        1170
AAT CCT CAG TTT GTG GTC TGC CAG CTA AAG GTG AAG ATA TAT TCC TCC AAT
 N   P   Q   F   V   V   C   Q   L   K   V   K   I   Y   S   S   N>

   1179        1188        1197        1206        1215        1224
TCA GGA CCC ACA CGA CGG GAA GAC AAG TTC ATG TAC TTT GAG TTC CCT CAG
 S   G   P   T   R   R   E   D   K   F   M   Y   F   E   F   P   Q>

       1233        1242        1251        1260        1269
CCG TTA CCT GTG TGT GGT GAT ATC AAA GTA GAG TTC TTC CAC AAA CAG AAC
 P   L   P   V   C   G   D   I   K   V   E   F   F   H   K   Q   N>

1278        1287        1296        1305        1314        1323
AAG ATG CTA AAA AAG GAC AAA ATG TTT CAC TTT TGG GTA AAT ACA TTC TTC
 K   M   L   K   K   D   K   M   F   H   F   W   V   N   T   F   F>
```

Figure 1B

```
   1332        1341        1350        1359        1368        1377
ATA CCA GGA CCA GAG GAA ACC TCA GAA AAA GTA GAA AAT GGA AGT CTA TGT
 I   P   G   P   E   E   T   S   E   K   V   E   N   G   S   L   C>

       1386        1395        1404        1413        1422
GAT CAA GAA ATC GAT AGC ATT TGC AGT ATA GAG CGT GCA GAT AAT GAC AAG
 D   Q   E   I   D   S   I   C   S   I   E   R   A   D   N   D   K>

1431        1440        1449        1458        1467        1476
GAA TAT CTA GTA CTT ACT TTA ACA AAA AAT GAT CTT GAC AAA GCA AAT AAA
 E   Y   L   V   L   T   L   T   K   N   D   L   D   K   A   N   K>

   1485        1494        1503        1512        1521        1530
GAC AAA GCC AAC CGA TAC TTT TCT CCA AAT TTT AAG GTG AAG CTG TAC TTC
 D   K   A   N   R   Y   F   S   P   N   F   K   V   K   L   Y   F>

       1539        1548        1557        1566        1575
ACA AAA ACA GTA GAG GAG CCG TCA AAT CCA GAG GCT AGC AGT TCA ACT TCT
 T   K   T   V   E   E   P   S   N   P   E   A   S   S   S   T   S>

1584        1593        1602        1611        1620        1629
GTA ACA CCA GAT GTT AGT GAC AAT GAA CCT GAT CAT TAT AGA TAT TCT GAC
 V   T   P   D   V   S   D   N   E   P   D   H   Y   R   Y   S   D>

   1638        1647        1656        1665        1674        1683
ACC ACT GAC TCT GAT CCA GAG AAT GAA CCT TTT GAT GAA GAT CAG CAT ACA
 T   T   D   S   D   P   E   N   E   P   F   D   E   D   Q   H   T>

       1692        1701        1710        1719        1728
CAA ATT ACA AAA GTC TGA ATT TTT TTT TAT CAA GAG GGA TAA AAC ACC ATG
 Q   I   T   K   V

1737        1746        1755        1764        1773        1782
AAA ATA AAC TTG AAT AAA CTG AAA AAA AAA AAA AAA AAA AGT GCG GCC GC
```

Figure 1C

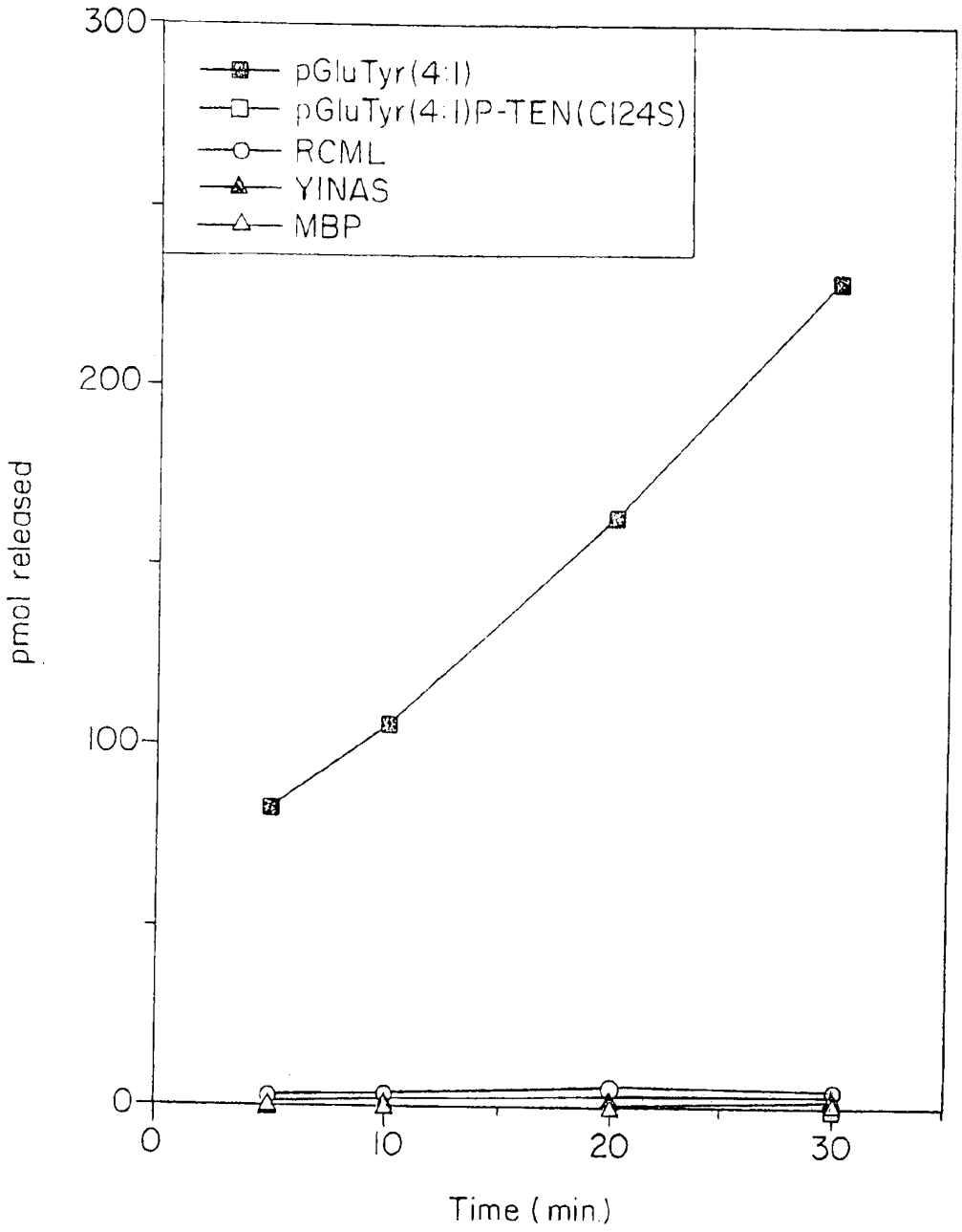
300
200
100
0
pmol released
pGluTyr(4:1)
pGluTyr(4:1)P-TEN(C124S)
RCML
YINAS
MBP
0
10
20
30
Time (min.)

FIG. 2A

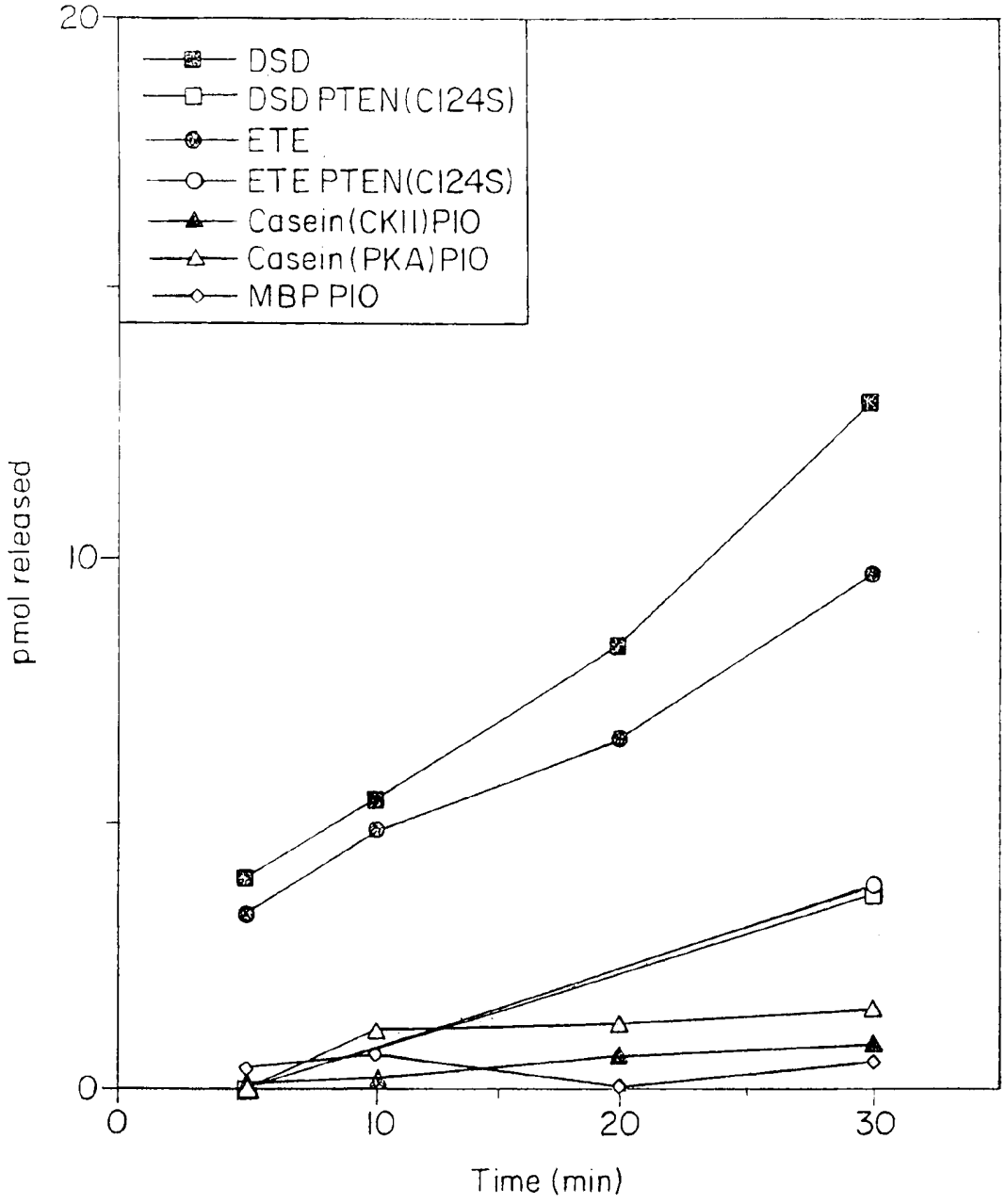
20
10
0
pmol released
DSD
DSD PTEN(C124S)
ETE
ETE PTEN(C124S)
Casein(CKII)PIO
Casein(PKA)PIO
MBP PIO
0
10
20
30
Time (min)

FIG. 2B

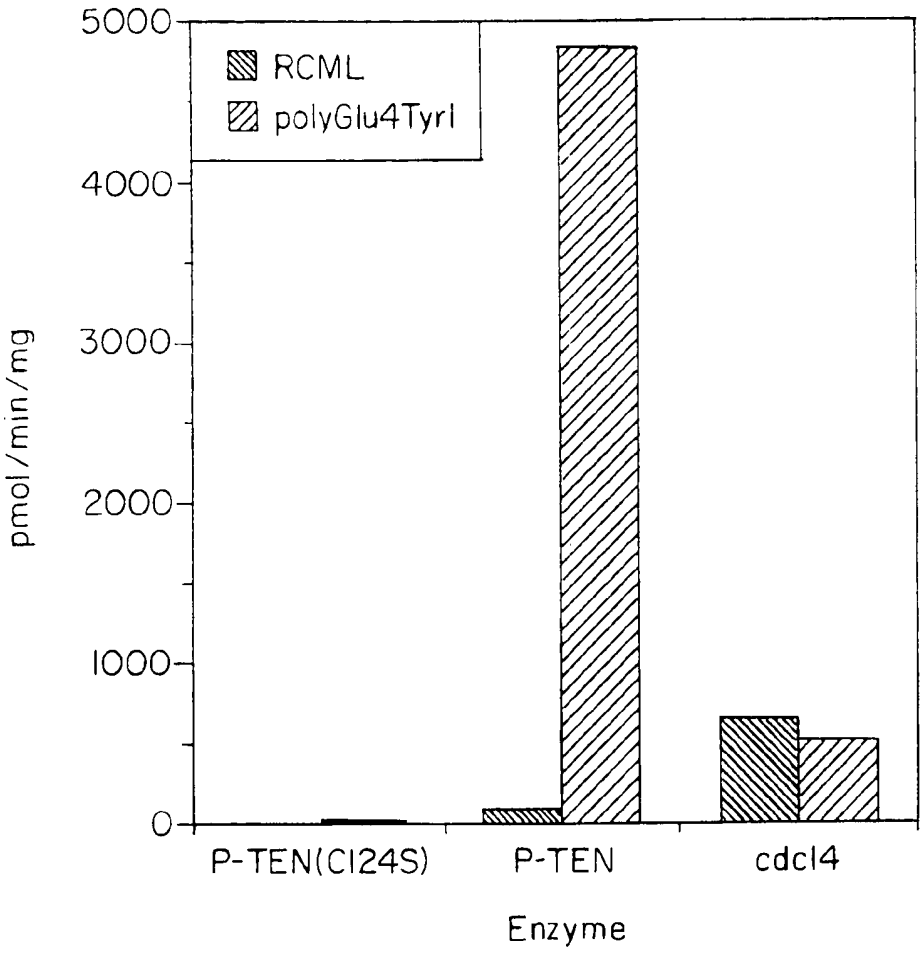
5000
4000
3000
2000
1000
0
pmol/min/mg
RCML
polyGlu4Tyr1
P-TEN(C124S)
P-TEN
cdc14
Enzyme

FIG. 2C

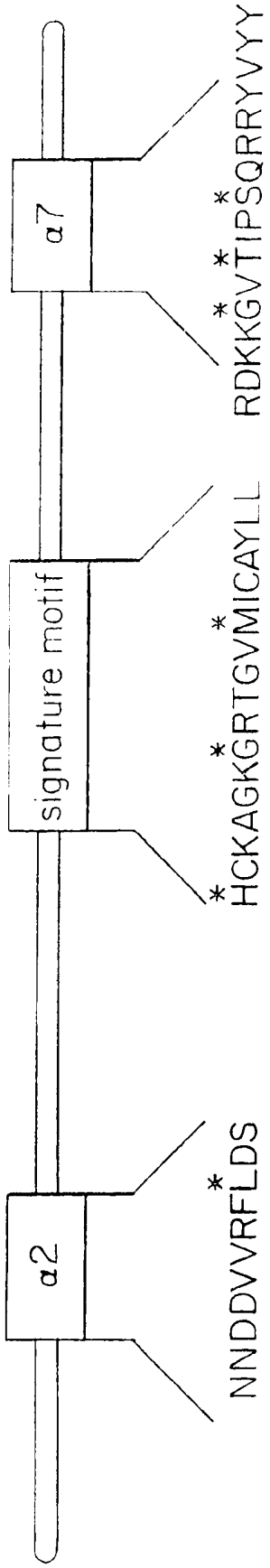
α7
signature motif
α2
RDKKGVTIPSQRRYVYY
HCKAGKGRTGVMICAYLL
NNDDVVRFLDS

FIG. 3

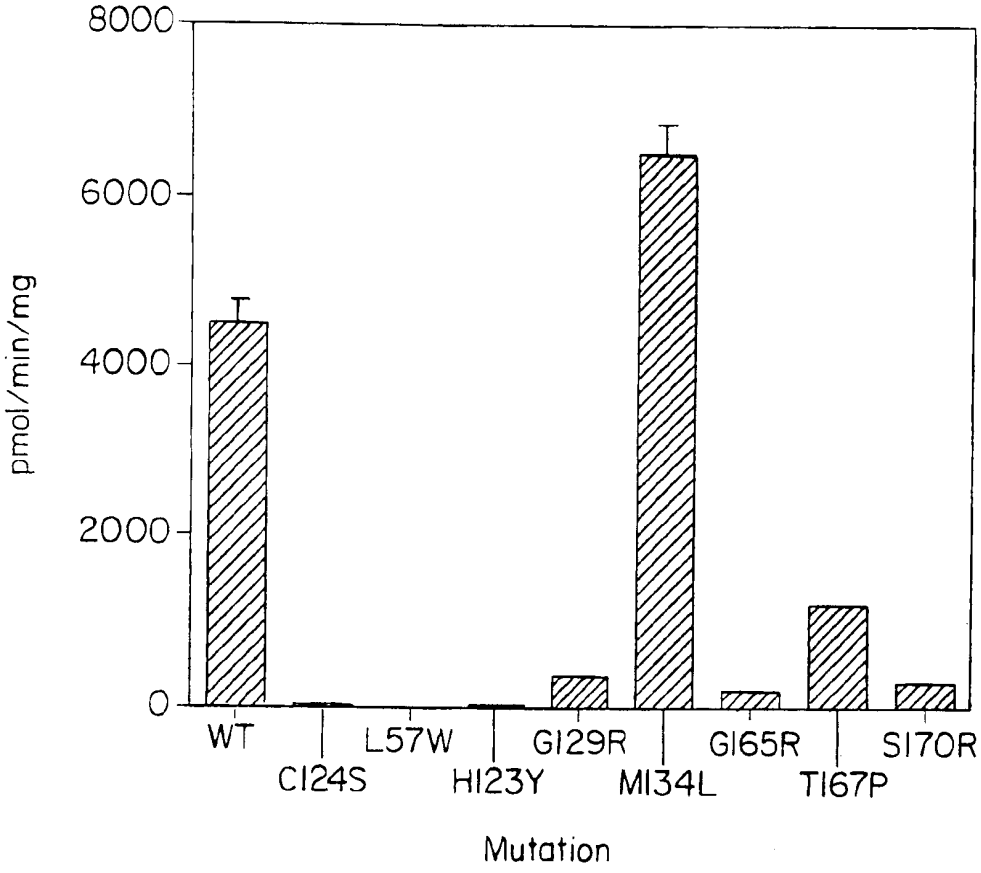
8000
6000
4000
2000
0
pmol/min/mg
WT
C124S
L57W
H123Y
G129R
M134L
G165R
T167P
S170R
Mutation

FIG. 4

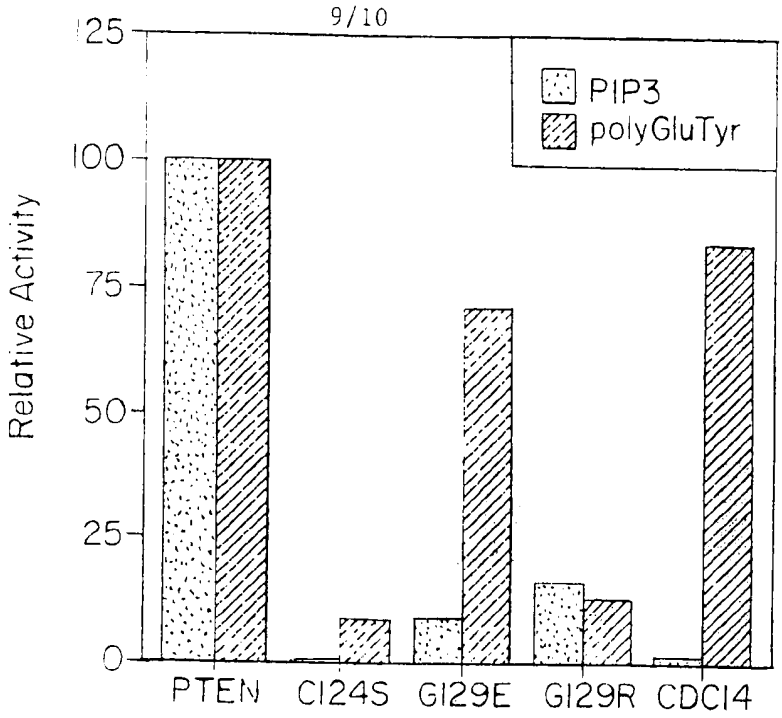
125
100
75
50
25
0
Relative Activity
PIP3
polyGluTyr
PTEN
C124S
G129E
G129R
CDC14

FIG. 5A

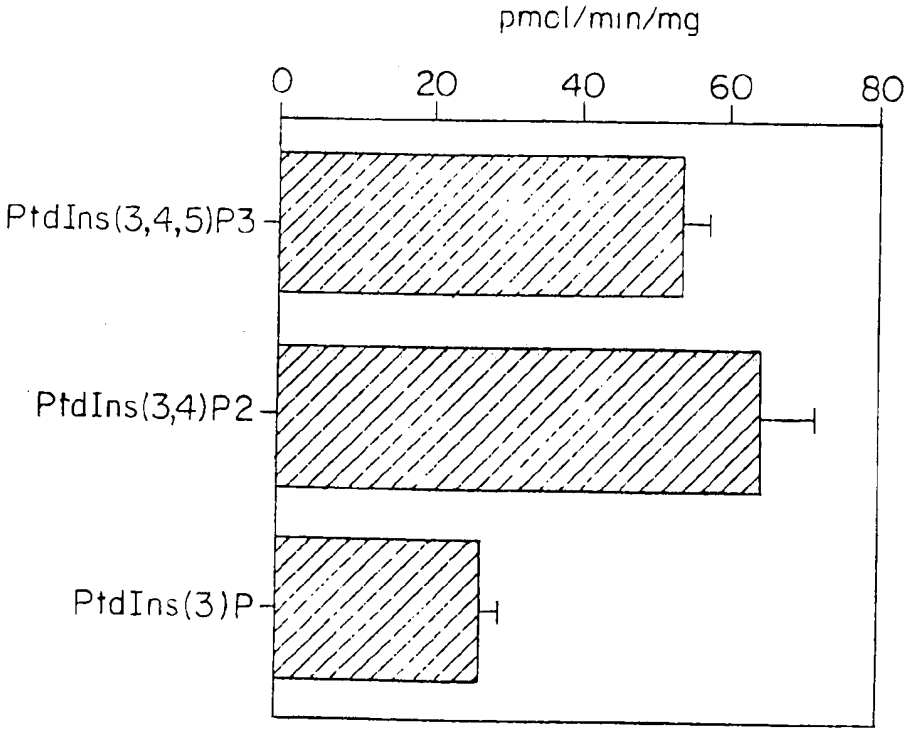
pmol/min/mg
0
20
40
60
80
PtdIns(3,4,5)P3
PtdIns(3,4)P2
PtdIns(3)P

FIG. 5B

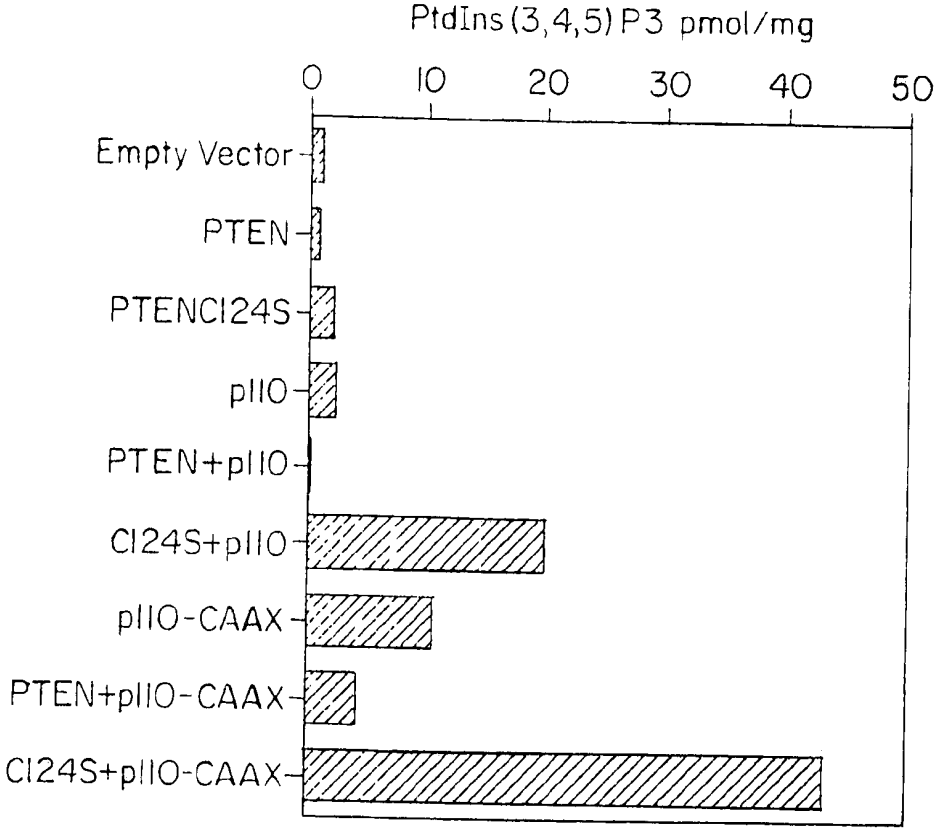
PtdIns (3,4,5) P3 pmol/mg
0
10
20
30
40
50
Empty Vector
PTEN
PTENCI24S
pI10
PTEN+pI10
CI24S+pI10
pI10-CAAX
PTEN+pI10-CAAX
CI24S+pI10-CAAX

FIG. 6

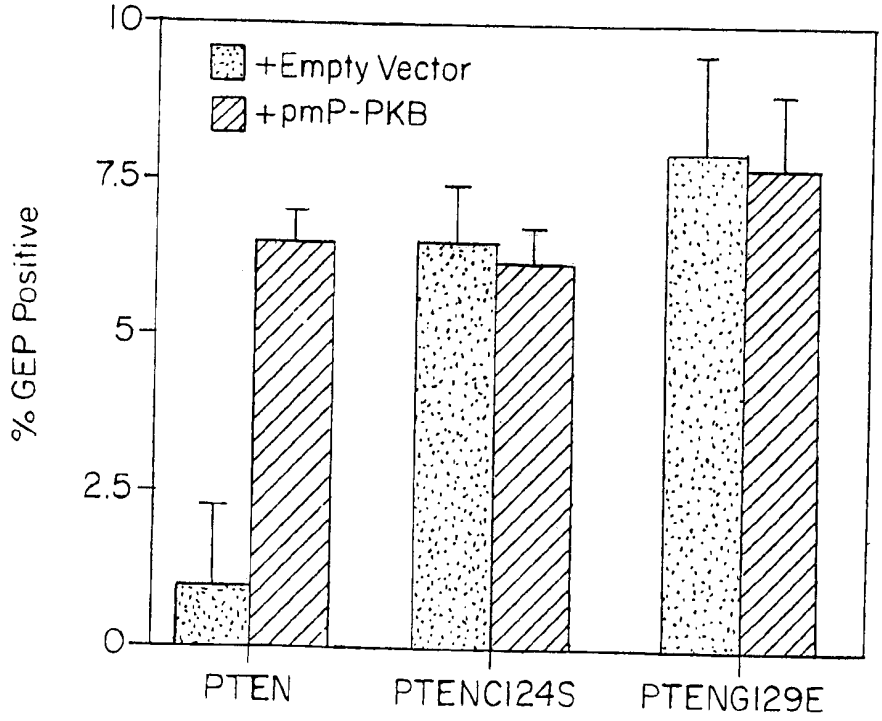
10
7.5
5
2.5
0
% GEP Positive
+Empty Vector
+pmP-PKB
PTEN
PTENCI24S
PTENGI29E

FIG. 7